Dietary phytochemical diversity to enhance health, welfare and production of grazing ruminants, while reducing environmental impact.

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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The objective of the outlined research was to determine how a fermented seaweed extract (SWO) and seaweed plus terrestrial plants (SWP) extract influenced ruminant health, productivity, environmental impacts (i.e. enteric methane and urinary N excretions), and the foraging ecology of young livestock. This was conducted over seven experiments. Chapter 3 implemented an *in vitro* methodology to determine the dose effect of SWO on fermentation parameters. The lowest dose implemented, which would relate to 5-mL/hd/d, reduced ammonia production by 6%, which may indicate lower urinary N excretions in vivo. Chapter 4 applied the determined dose (5-mL/d) to dairy cows during the last third of gestation with either SWO, SWP, or water (CON), and then the dose was increased to 100-mL per d after calving during the early lactation portion of the experiment. It was determined that the cows receiving SWO and SWP had reduced oxidative stress prior to calving and lower oxidative and metabolic stress 3-d after calving compared with the CON cows. Cows provided SWP after calving had 18% lower urinary N excretion, which is described in chapter 5. In chapter 6, pregnant ewes were either provided no supplement (CON-) or a grain based supplement with no plant extracts (CON+), with SWO (10-mL/hd/d), or SWP (10-mL/hd/d). At peak lactation (28 days-in-milk) grain supplementation (CON+) increased oxidative stress compared with CON-, and this effect was negated by SWO and SWP. Grain supplementation has been shown to induce oxidative stress in several animal models at peak lactation. In chapter 7, ewes were managed to lamb as yearlings, with their offspring precluded from consuming the same treatment supplements as in chapter 6. While SWO and SWP showed no benefit to oxidative stress of the ewes, possibly due to the low metabolic stress experienced, the lambs whose dams were provided SWO and SWP had lower oxidative stress one day after weaning. This indicates a greater maternal transmission of antioxidants from the SWO and SWP dams providing defense against oxidative stress induced from the physiological stress associated with weaning. The ram lambs born during chapter 6 continued to receive the supplement treatments of their dams until the start of the experiment described in chapter 8. The ram lambs were allocated to spatially separated strips sown to ryegrass (*Lolium perenne*), chicory, plantain, lucerne, and dock. Previous exposure to SWP reduced dietary neophobia, whereas the SWO and CON lambs exhibited substantial dietary neophobia to chicory and plantain. For chapter 9, the lambs born during chapter 7 were placed in the same paddocks as the ram lambs used in chapter 8. Similar to chapter 8, it was determined that lambs born to ewes provided SWP showed less dietary neophobia to chicory and lucerne compared with the lambs born to ewes provided SWO and CON. This chapter determined that dietary experience was obtained either from in utero or maternal milk exposure. Collectively, these experiments show the benefit of fermented plant extracts to improve animal health, reduce environmental impacts, and how foraging decisions of ruminants can be manipulated by previous exposure.

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Chapter 1

Introduction

Plant Plant secondary compounds (PSC) benefit plants by providing deterrent to herbivory, antibacterial and antifungal, UV light protection, attraction to pollinators, allelochemicals, and others properties. Over ingestion of PSC by livestock though reduces digestibility, intake, health and thereby animal performance. On the contrary, consumed in appropriate amounts, these compounds may provide nutraceutical, pharmaceutical, prophylactic, and therapeutical benefits to the animals (Provenza et al. 2015). In nature, dietary and phytochemical diversity is the rule, not the exception, and ruminants use it to their benefit (Provenza et al., 2015; Gregorini et al., 2017). Moreover, certain PSC reduce enteric methane (CH_4) production and nitrogen excretion, as well as increase the supply of true protein and amino acids to the duodenum (Waghorn, 2007). Dietary monotony of productive systems constrain ruminants choice and their capability to benefit from diverse diets; all of which reduces animal health, welfare, performance and nutrient use efficiency, as well as increase environmemntal impacts (Gregorini et al, 2017).

Seaweed has been used by the Greeks in animal agriculture since 45 B.C. (Evans and Critchley, 2014). Seaweed extracts (mainly *Ascophyllum nodosum*) have been examined in the recent past, showing to be a good source of phlorotannins (Evans and Critchley, 2014) and benefiting cattle (Leupp et al., 2005). *A. nodosum* extract applicated on swards have also shown benefits in forage growth and digestibility, antioxidant activity, and pro- and pre- biotic effects (Fike et al., 2001; Leupp et al., 2005). Currently, in New Zealand there is a renewed interest in the use of seaweed derived product for animal health, which is based on the extraction of *Ecklonia radiata*, a species native to New Zealand (AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand). Despite these benefits, and the growing anecdotal evidence, there is still a lack of scientific information on the use and animal benefits of seaweed extracts

and its combination with other PSC bioactives as source or way to increase dietary phytochemical diversity and there by nutraceutical, pharmaceutical, prophylactic, and therapeutical benefits to grazing ruminants.

The proposed research focuses on the prophylactic, therapeutical, pharmaceutical, and nutriceutical properties of phytochemically rich and diverse diets, using seaweed and seaweed plus terrestrial sources of plant bioactives to supplement ryegrass based diets using different ruminant models. Following this introduction, this thesis is composed of a literature review which has been accepted in an international peer-reviewed journal (Chapter 2), seven experiments described in manuscripts which have been accepted, are in review, or are in preparation for submission to international peer-reviewed journals (Chapters 3-9), a discussion chapter which is in review in an international peer-reviewed journal, and finally a general discussion. It is hypothesized that the provision of rich arrangements of plant secondary compounds will: 1) alter rumen function, increase nutrient use efficiency and reduce environmental impact in terms of CH_4 production and yield, and urinary N excretion; 2) reduce oxidative stress in lactating and stressed livestock; and 3) alter foraging behaviour through providing dietary experience through these plant extracts during early life and through maternal transmission. Hypothesis number one is first explored in Chapter 3 using *in vitro* methods exploring the ability of plant extracts to reduce rumen ammonia production and later in Chapters 4 and 5 using dairy cattle where rumen fermentation characteristics, enteric gas emissions, and urinary N excretions are explored. The second hypothesis is tested with periparturient dairy cattle in Chapter 4 and with lactating mature and yearling ewes in Chapters 6 and 7, respectively, where the antioxidant benefits of the plant extracts are assessed. The third and final hypothesis is tested in Chapter 8 where the effects of early life experience to the plant extracts on dietary preference of novel forages is tested and in Chapter 9 where the maternal transmission of dietary experience is assessed.

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Chapter 2

Literature Review

2.1 Abstract

Ruminants evolved in diverse landscapes of which they utilized, by choice, a diverse arrangement of plants (grasses, forbs, and trees) for food. These plants provide them with both primary (carbohydrates, protein, etc.) and secondary (phenolics, terpenes, etc.) compounds (PPC and PSC, respectively). As no one plant could possibly constitute a “balanced-diet”, ruminants mix diets so that they can exploit arrangements of PPC to meet their individual requirements. Diet mixing also allows for ruminants to ingest PSC at levels, acquiring their benefits such as antioxidants and reduced gastrointestinal parasites, without overstepping thresholds of toxicity. Meeting dietary requirements is assumed to provide satisfaction through achieving positive internal status and comfort, thereby a sense of hedonic (happiness through pleasure) wellbeing. Furthermore, choice including dietary choice is a factor influencing wellbeing of ruminants in a manner akin to that in humans. Choice may facilitate eudaimonic (happiness through pursuit of purpose) wellbeing in livestock. Nutritional status plays an integral role in oxidative stress, which is linked with illness. Several diseases in livestock have been directly linked to oxidative stress. Mastitis, metritis, hypocalcaemia, and retained placenta occur in animals transitioning from dry to lactating and have been linked to oxidative stress and such a stress has likewise been linked to diseases that occur in growing livestock as well, such as bovine respiratory disease. The link between physiological stress and oxidative stress is not well defined in livestock but is evident in humans. As dietary diversity allows animals to select more adequately balanced diets (improved nutrition), take advantage of PSC (natural antioxidants), and allows for choice (improved animal wellbeing) there is a strong possibility for ruminants to improve their oxidative status and thus health, wellbeing, and therefor production. The purposes of this review are to first, provide an

introduction to oxidative and physiological stress, and nutritional status as effected by dietary diversity, with special attention to providing support and on answering the "how". Second, to provide evidence of how these stresses are connected and influence each other, and finally discuss how dietary diversity provides a beneficial link to all three and enhances both eudaimonic and hedonic wellbeing.

Keywords: Grazing, animal welfare, taxonomical diversity, biochemical diversity

2.2 Introduction

Dietary diversity in ruminants has recently received considerable attention in the literature (Scott and Provenza, 1999; Atwood et al., 2001; Provenza et al., 2003; Manteca et al., 2008; Villalba et al., 2010; Villalba et al., 2015b; Villalba et al., 2015a; Gregorini et al., 2017). Much of this work has focused on how dietary diversity can improve animal production by providing animals with the opportunity to choose and mix their diets. By doing so, the animals are better able to meet their individual requirements and self-medicate, acquiring nutraceuticals, pharmaceuticals, and prophylactic benefits associated with the ingestion of specific secondary compounds (PSC) at self-regulated safe levels of intake (Provenza, 1996). Here we use the term wellbeing when discussing the subjective mental state of the animal and welfare as the animal state including wellbeing, health and the animals experience with their environment. In a comprehensive review article on dietary diversity and welfare, Manteca et al. (2008) concluded that the improved nutritional status given by appropriate supply of plant primary compounds (PPC) and the improved health benefits by the PSC are indicative of the intimate relationship between dietary diversity and animal welfare. However, the benefits of dietary diversity on animal welfare have been discussed only as they relate to hedonic wellbeing. The word hedonic stems from the Greek word *hēdone*, meaning pleasure, and thus hedonic wellbeing is the balance between positive and negative emotions (Deci and Ryan, 2008). Emotions are clusters of experiences related to health, fear, nutritional comfort, nutrient supply, and familiarity, as a few examples. Animals integrate those experiences, at different time scales forming either positive or negative emotions (Boissy and Erhard,

2014). Another concept of wellbeing, commonly applied to humans, is eudaimonia, which was first proposed by Aristotle. Eudaimonia stems from the Greek words *Eu*, for good, and *daimon*, for guardian. There are several definitions proposed for eudaimonia, but the one which we propose can best be applied to ruminants is one of function. We propose that eudaimonic wellbeing is achieved in livestock and other animals when they are able to pursue their potential (Deci and Ryan, 2008). To that end, eudaimonic wellbeing is achieved when a subject achieves its telos, which is defined as a given purpose (Harfeld, 2013). Eudaimonic wellbeing has rarely been applied to livestock welfare but Harfeld (2013) proposed that an animal's telos is enshrined in the species' uniqueness which is genetically coded (see also Nordenfelt 2011). We propose that telos may also be considered as an individual trait and this is supported by individual animal personalities, by genetically related grazing personalities in ruminants (Bailey et al., 2015; Gregorini et al., 2017), and by the reduction of stress when choice is allowed (Owen et al., 2005; Manteca et al., 2008). Improved wellbeing by offering choice to animals both facilitates and provides evidence in support for eudaimonia and telos in livestock, as it has been suggested that without choice one cannot pursue their telos and thus achieve eudaimonic wellbeing (Harfeld, 2013). Even if the available options (e.g. dietary options) provided will only allow the animals to choose the least-worse option available for their individual needs, we argue eudaimonic wellbeing will be improved.

We hypothesize that merely providing choice would improve eudaimonic wellbeing in livestock; however, for dietary diversity to improve hedonic wellbeing there must first be some subsequent actions to increase pleasure or reduce negative experiences and thereby emotions. Such actions constitute responses to environmental stimuli that provoke oxidative stress, physiological stress, or reduced nutritional state of the animal. These three features of animal state are of interest with regard to welfare and hence production. Oxidative stress influences the pathophysiology of diseases, and its management has received much attention (Sordillo et al., 2009; Sordillo and Aitken, 2009; Celi, 2010; Celi and Gabai, 2015). Physiological stress including cortisol release is often used as an index of welfare (Dantzer and Mormède, 1983), which in turn is linked to production and economic return (Fraser, 2008). Appropriate

nutrition for each respective class of livestock is obviously a major feature of every livestock production system.

In this review we describe and explain how the influence of oxidative stress, physiological stress, and nutritional state influence wellbeing of grazing livestock as a response to taxonomic and biochemical diversity of the diet. We present a conceptual model (Figure 2.1) describing the interactive links between dietary diversity and animal state, resulting in positive effects on animal health and wellbeing (both hedonic and eudaimonic).

2.3 Oxidative Stress

Oxidative stress is a state of imbalance between oxidants (e.g. reactive oxygen metabolites) and antioxidants (both enzymatic [e.g. superoxide dismutase] and non-enzymatic [e.g. vitamin E and glutathione]; Bernabucci et al., 2005; Jin et al., 2014). The circulating level of oxidants is subject to homeostatic regulation but situations may occur in which the animal is exposed to stressors, such as high metabolic demand, gastrointestinal parasites, heat stress, and diseases (Celi and Gabai, 2015), which cause the rate of production of oxidants to exceed the capacity of the homeostatic regulatory system. The remaining oxidants damage important biological molecules (including lipids, proteins, DNA, and RNA; Celi and Gabai, 2015), which then lead to metabolic and pathological disorder (Lykkesfeldt and Svendsen, 2007).

An example of this is isoprostane production, which has similar actions as prostaglandins (e.g. prostaglandin F-2α). Prostaglandins are involved in the regulation of many physiological functions (e.g. pregnancy maintenance) and also in inflammation and immune responses (Basu, 2010). The key enzymes involved in the conversion of arachidonic acid to eicosanoids (e.g. prostaglandins) is cyclooxygenases (Basu, 2010). In cattle, prostaglandin F2α are an important part of the estrus cycle as they cause luteolysis (degradation of the corpus luteum; Odde, 1990). Prostaglandins are also important in the pathological manifestation of chemical or physical injury, in fact nonsteroidal anti-inflammatory drugs function by inhibiting prostaglandin synthesis, by blocking the cyclooxygenases (Chand and Eyre, 1977).

Similar compounds to prostaglandins, the isoprostanes, are generated independently of cyclooxygenase enzymes through the peroxidation of arachidonic acid by oxidants (Montuschi et al., 2007). Isoprostanes have been identified as a promising in vivo marker for oxidative stress and they have been found to have negative biological effects as they can bind to many of the same receptors as cyclooxygenase derived prostaglandins (Montuschi et al., 2007). These effects include vasoconstriction and airway constriction, and therefore may be pathophysiological mediators of oxidative damage (Montuschi et al., 2007). Thus, isoprostanes are formed through oxidants oxidizing a biological molecule (arachidonic acid) and subsequently inducing inflammatory responses to oxidative damage. We postulate that the damage to biological compounds leading to metabolic or pathological disorders and inflammation, such as arachidonic acid, would result in discomfort and subsequently reduce wellbeing. This is supported by some works who found a positive correlation between blood cortisol and isoprostane concentrations (Kasimanickam et al., 2018; Kasimanickam et al., 2019). The integration of uncomfortable experiences leads to negative emotions, thus reducing hedonic wellbeing. The link between oxidative and physiological stress is discussed further below.

2.3.1 Physiological Causes of Oxidative Stress

The following paragraphs provides a summary of the biochemical sources of oxidants, which is the "how" behind oxidative stress. This detail is important because it provides background information for understanding how biochemical (PSC) diverse diets reduce oxidative stress in grazing ruminants. We later describe how the improved antioxidant status of the animals would lead to enhanced hedonic wellbeing.

Oxidants are important in several physiological and biochemical reactions; consequently, they are well managed by the body. For example, Superoxide ($O_2^{\bullet-}$) is an oxidant produced in the mitochondria of mammalian cells, which is subsequently converted to H_2O_2 by mitochondrial superoxide dismutase (Muller, 2000; Murphy, 2009). This $O_2^{\bullet-}$is generated in the electron transport chain, with the majority being produced by complex I, and a negligible amount by complex III (Murphy, 2009). These $O_2^{\bullet-}$ are

converted to H_2O_2, which exits the mitochondria to act as a redox signal to the cellular cytosol and the nucleus. Hydrogen peroxide, while still an oxidant, has a lower second order rate constant for reactions with biomolecules than other oxidants (e.g. hydroxyl radical or $O_2^{\bullet-}$) and is therefore appropriate for redox signaling (Sies et al., 2017). These redox reactions are important in regulation of enzymes and transcription factors (Abate et al., 1990; Sies et al., 2017). This elicits various cellular responses such as enzyme activity, substrate supply, and mitochondrial biogenesis (Hurd et al., 2007; Murphy, 2009). Redox signaling shows how oxidant production, when under normal physiological functions, is necessary rather than negative.

Another physiological source of oxidants come from phagocytic cells removing foreign organisms. Reactive oxygen species are toxic to many microorganisms. When phagocytic cells (e.g. neutrophils) engulf bacteria there is an initial oxygen consumption (called oxidative burst) where an NADPH oxidase complex transfers electrons from NADPH to oxygen in order to generate superoxide. As some works have reported that superoxide does not kill bacteria, it is believed that additional secondary oxidants are generated and responsible for bacterial death (Hampton et al., 1998). One important example of phagocytic cells to ruminants is neutrophils involvement in removing pathogens related to pneumonia such as Mannheimia haemolytica. Removal of these pathogens is reliant on active immunity and the innate immune function, such as neutrophils (Ackermann and Brogden, 2000). Therefore, oxidant creation in this context is required by the body to remove foreign organisms and to maintain health and comfort through the relief from pain, and thus hedonic wellbeing.

The final source of oxidants to be discussed in this review occurs in the gastrointestinal tract. Halliwell et al. (2000) proposed several sources of gastrointestinal derived oxidants. Firstly, foods will generally contain iron, often in the insoluble Fe_3^+ salt form. Gastric acid can solubilize ferric and metallic iron. The Fe_3^+ is then reduced to Fe_2^+, which is easier to absorb with stimulation by ascorbate. The oxidant, hydroxyl radical can be produced when ascorbate and Fe_2^+ are mixed, without H_2O_2 (Fenton Chemistry). Similar reactions can occur from Cu_2^+ and ascorbate (Halliwell et al., 2000). Other sources

include: haem (from haem proteins), dietary lipids that undergo peroxidation, foods containing isoprostanes, oxidized cholesterol, nitrites, the gastrointestinal immune system, and oxidized phenolic compounds such as hydroxyhydroquinone (from coffee; Halliwell et al., 2000). This may indicate that oxidized phenolic compounds from forage plants may act as oxidants. While information from the human literature is abundant, to our knowledge, few works have reported how important ruminant gastrointestinal derived oxidants are. One experiment measured antioxidant activity of rumen fluid and plasma from faunated or defaunated rumens (with or without protozoa; Gazi et al., 2007). It was found that faunated rumens had greater antioxidant activity than defaunated rumens in both ruminal fluid and in the plasma (Gazi et al., 2007). Increased antioxidant capacity in the rumen leading to increased antioxidant capacity in the plasma would indicate that rumen fluid is important to the whole animal's antioxidant status. However, we are unaware of any research showing how this would translate to the lower gastrointestinal tract, but due to research on humans (see Halliwell et al., 2000 for a review), we speculate that this is a significant source of oxidant production which requires further investigation.

2.4 Defence Mechanisms Against Oxidative Stress

2.4.1 Enzymatic Defence Against Oxidative Stress

Antioxidant enzymes are a major intrinsic, or endogenous, oxidant defence. Superoxide dismutase (SOD) is found in the cytoplasm and the mitochondria of cells in the Cu-SOD and Mn-SOD forms, respectively (Harris, 1992). This enzyme converts the superoxide anion (which is highly radical) to hydrogen peroxide (H_2O_2; which is less radical). Glutathione peroxidase (GPx) is then responsible for converting H_2O_2 to water and oxidized glutathione. Catalase is another important antioxidant enzyme which converts H_2O_2 to water and O_2. Glutathione reductase then 'recycles' the oxidized glutathione by reducing it to its active form, reduced glutathione. This reaction occurs by oxidation of NADPH to $NADP^+$ by GR (Mirończuk-Chodakowska et al., 2018).

As these enzymes are important in maintaining homeostasis of ruminants, their quantification in biological samples have been identified as a marker of oxidative stress (Celi, 2011). However, their

interpretation is not always straight forward. On one hand, when supplemented with selenium, GPx levels in ruminant erythrocytes increase, which is expected as GPx is a selenium dependent enzyme (Gerloff, 1992). These experiments interpret this result as an improvement in antioxidant status. On the other hand, there are greater levels of antioxidant enzymes in erythrocytes of dairy cows in the summer than in spring, which is due to increased heat stress (Bernabucci et al., 2002). These increases are due to increased oxidative stress, as it is known that heat stress causes oxidative stress (Bernabucci et al., 2002; Chauhan et al., 2014). Due to these inconsistencies, we recommend implementing multiple markers of antioxidant status in order to assist with interpretation.

2.4.2 Intrinsic Non-Enzymatic Defense Against Oxidative Stress

Non-enzyme antioxidants such as glutathione, uric acid, melatonin, bilirubin, polyamines, and metal binding proteins are also a part of the intrinsic oxidative stress defence system (Mirończuk-Chodakowska et al., 2018). While important, this review will not delve into detail on them. Mirończuk-Chodakowska et al. (2018) provides a detailed review on non-enzymatic, intrinsic antioxidants. One example of non-enzymatic antioxidants is albumin, which is important to grazing ruminant health. Albumin is the major antioxidant in circulating blood, which is continuously exposed to oxidative stress (Roche et al., 2008). In ruminants, albumin has been found to be incorporated into colostrum and milk (Levieux and Ollier, 1999). Thus, albumin provides antioxidant defense in several biological fluids, such as blood, colostrum, and milk. In the following section we will go in depth on how a diverse diet providing biochemical diversity in plant secondary compounds can provide extrinsic antioxidant defense.

2.5 Dietary Diversity and Antioxidant Defense

Extrinsic (exogenous or dietary antioxidants) defenses against oxidative stress come from food. These antioxidants include vitamins E and C and PSC, such as phenolics, terpenes and terpenoids. When offered an array of forages animals select and consume natural antioxidants at rates below toxic levels of intake (Acamovic and Brooker, 2005; Waghorn, 2008). Plant secondary compounds, especially phenolic compounds, have been shown to improve antioxidant status and reduce plasma levels of oxidative

components. Phenolic and polyphenolic compounds (tannins and flavonoids, from terrestrial plants; phlorotannins, from aquatic plants [seaweeds]) can have free radical scavenging properties. Phenolic compound containing-extracts from the common daisy (Bellis perennis L.) showed free-radical scavenging activity of 2,2-Diphenyl-1-picrylhydrazyl in vitro (Karakas et al., 2017). This ability was likewise demonstrated with isolated flavonoids from Opuntia monacantha (Valente et al., 2010). Additionally, Chakraborty et al. (2015) extracted phlorotannins from three species of red seaweed (Division: Rhodophyta) and saw marked reductions in free radicals. This antioxidant activity can have remarkable effects on antioxidant status when applied to plants and animals (Allen et al., 2001; Luciano et al., 2011). Kannan et al. (2007) reported increased antioxidant enzymes and reduced lipid peroxidation when sheep were treated with a seaweed extract and challenged with transportation stress. Milking goats provided tannins from sulla (Sulla coronarium L.) forage, had improved plasma antioxidant capacity (Di Trana et al., 2015). Sheep provided plant by-products (tomato pomace and grape skin) had upregulated transcriptional activity to genes that are involved in oxidant defence enzymes (Sgorlon et al., 2006). When transition dairy cows were provided tannins from chestnut there were lower plasma and milk malondialdehyde (MDA; a marker of lipid peroxidation) and increased antioxidant enzyme activities in plasma and the liver (Liu et al., 2013). These experiments, and others, highlight the potential of PSC to improve antioxidant status, which would result in a better internal state and improve hedonic wellbeing of grazing ruminants.

2.6 Plant Secondary Compounds as Antioxidants: Potential Modes of Action

Several modes of action exist for PSC, especially phenolic compounds, to exhibit antioxidant activity. One mode would be by providing antioxidant activity directly in bodily fluids and tissues. In order for this to occur, antioxidant PSC would need to be absorbed and incorporated into tissues (Vasta and Luciano, 2011). Evidence for this can be seen by the increased product quality, such as improved shelf life, color stability, flavor, and odor, from animals provided PSC seen by several experiments (Vasta and Luciano, 2011). One experiment provided sheep plant extracts, including rosemary (*Rosemarinus*

officinalis), grape (*Vinis vitifera*), citrus (*Citrus paradise*), and marigold (*Calendula officinalis*; Gladine et al., 2007). It was found polyphenolic compounds, including condensed tannins from grapes, are catabolized to monomeric phenolics, become bioavailable, and were present in the blood of the sheep. It was also reported that naringin from the citrus extract was found in the plasma, which is contrary to what occurs in monogastrics (Gladine et al., 2007). Another work reported that the ultraviolet-absorbing compounds in milk result from ingested phenolic compounds from forages (various hays, silages, and fresh pasture; Besle et al., 2010). Additionally, when supplied tannins from sulla, goat milk was found to have greater phenolic compounds and total antioxidant capacity (Di Trana et al., 2015). These experiments indicate that antioxidant PSC such as phenolics can be absorbed from ruminant gastrointestinal tracts and incorporated into milk products thus improving product quality, but also potentially exerting nutraceutical, pharmaceutical and prophylactic activity.

Plant secondary compounds have also been measured in meat products. When ewes were dosed rosemary (*Rosmarinus officinalis*) extract, their offspring were found to have increased phenolic compounds incorporated in their meat at slaughter (Moñino et al., 2008). This incorporation in the tissue increased the antioxidant capacity of the meat (Moñino et al., 2008). In a similar experiment, Nieto et al. (2011) gave pregnant ewes either 0, 10, or 20% of their diet with distilled rosemary leaf and observed delayed lipid oxidation, odor, and flavor spoilage of their lamb's meat due to the additions. In another experiment, rosemary leaf distillate additions to pregnant ewes improved lamb meat quality characteristics (Nieto et al., 2010a). These results were corroborated when ewes were provided varying rates of thyme (*Thymus zygis* ssp.) leaves in their diet. Again, the antioxidant additions to their dam improved product quality and shelf life of the lamb meat (Nieto et al., 2010b). When lambs received a diet containing quebracho (*Schinopsis lorentzii*) tannins there was a 31.29 and 16.81% increase in total phenols and antioxidant capacity in the muscle compared to the control, respectively. The increased antioxidant status improved meat color stability (Luciano et al., 2011). Similarly, when growing chickens were provided a by-product of the olive oil industry (semi-solid olive cake; "pate"), meat oxidative

stability was improved and tyrosol and metabolites of hydroxytyrosol (phenolic compounds) were detected (Branciari et al., 2017). These results support the mode of action for a direct antioxidant activity at the bodily fluids and tissue level by absorbed PSC and this interpretation has also been suggested by Vasta and Luciano (2011).

Another potential mode of action for PSC is by providing antioxidant action in the gastrointestinal tract. As discussed above, the gastrointestinal tract is a major source of oxidants. This effect in livestock, to our knowledge, is largely unexplored. However, when sheep had long-term exposure to dietary heavy metals, it was found that there was oxidative damage to the gastrointestinal tract and concluded that lipid peroxidation was one of the mechanisms behind chronic heavy metal poisoning in ruminants (Faix et al., 2005). In humans, PSC, such as phenolics, have been found to alleviate or prevent gastrointestinal diseases such as ulcers (Repetto and Llesuy, 2002). Evidence of antioxidant benefits of PSC in ruminant's gastrointestinal tract is lacking and requires further research; however, as stated above we speculate that the ruminant gastrointestinal tract is a major source of oxidants and postulate that antioxidant PSC would alleviate this production.

Finally, PSC have been found to regulate gene expression to alter antioxidant status. The nuclear factor erythroid 2-related factor 2 (Nrf2) has been identified as the leading transcription factor behind oxidative stress defense (Ma, 2013). Nuclear factor erythroid 2-related factor 2 reduces oxidative stress directly by increasing antioxidant enzyme activity, regenerating oxidized cofactors (e.g. GSSG to GSH), synthesizing these reducing factors (e.g. GSH), and by increasing expression of antioxidant proteins (Ma, 2013). Plant secondary compounds have been shown to activate Nrf2, resulting in increased antioxidant enzymes in farm animals (Lee et al., 2017). An in vitro experiment on bovine mammary epithelial cells showed potential of tea polyphenolics to reduce oxidative stress when challenged by hydrogen peroxide, and that these results were due to upregulation of Nrf2 (Ma et al., 2018).

Oxidative stress is an important aspect of ruminant management. Reactive oxygen metabolites are both necessary for normal physiological functioning (e.g. redox signaling) but also, when produced at

levels that outpace the animal's defense system, can cause negative effects after they damage various biological molecules. The defense system in place for ruminants to handle oxidants are intrinsic antioxidant enzymes and non-enzyme antioxidants, but also extrinsic dietary antioxidants. Plant secondary compounds, which can be commonly found in many forages used in grazing ruminant production systems, provide an interesting opportunity to manage oxidative stress in grazing ruminants as they have several modes of actions. They can remove oxidants in the gastrointestinal tract, after absorption in the small intestine, by being incorporated into milk and tissues, and by regulating gene expression.

2.7 Physiological Stress and Hedonic and Eudaimonic Wellbeing

Physiological stress is the hormonal response that an organism experiences in response to a stressor, whether abiotic or biotic. Physiological stress manifests itself in the "fight or flight" response in organisms (Dantzer and Mormède, 1983). This response is elicited by the release of glucocorticoids (GC). Glucocorticoids, such as cortisol, have been studied as a marker of animal welfare with less cortisol acting as a marker for positive welfare (Dantzer and Mormède, 1983). Abiotic stressors include climatic events (e.g. heat stress). Biotic stressors are elicited from the animal's peers, predators and other animal species, animal handling (Grandin, 1997), and, more recently suggested, dietary monotony (Manteca et al., 2008; Villalba et al., 2010; Villalba et al., 2012; Gregorini et al., 2017). Dantzer and Mormède (1983) reviewed the causes of physiological stress and physiological pathway, from stress perception to hormonal responses. In brief, following the experience of a stressor, glucocorticoids (GC) are released following the hormonal cascade from the hypothalamic-pituitary axis (Dantzer and Mormède, 1983). In essence GC prepare the animals for the "fight or flight" through several metabolic responses. These include increased gluconeogenesis, reduced glucose uptake by the periphery, suppress insulin, and mobilize energy stores. Additionally, GC can alter behaviour and elicit anxiety behaviour (e.g. stereotypies; Dantzer and Mormède, 1983).

Historically, objective assessment of animal welfare has been done by measuring GC in the blood (Dantzer and Mormède, 1983). As animal handling to take the blood sample causes a stress response, it has been suggested that fecal cortisol metabolites (Palme et al., 1999) or hair (Tallo-Parra et al., 2014) cortisol levels are more accurate. While cortisol is the most predominant biomarker of welfare, there are several other markers available (see Barrell, 2019 for a recent review). However, most methods of measuring welfare would only provide insight on the negative state of the animal and it is often assumed that less cortisol provides insight into positive welfare, which may not always be the case (Yeates and Main, 2008). This necessitates research into objective markers of positive welfare. Some markers of positive welfare that have been suggested are vocalizations, measurements of neurotransmitters such as endorphins and dopamine, and hormones like oxytocin and serotonin (Boissy et al., 2007; Yeates and Main, 2008). While physiological stress and negative welfare may often be negatively correlated with positive welfare, it is time for the development of standardized methodologies for measuring positive states of animals.

Ethical management of animals has been predominately based upon the "five freedoms". These include the freedom from 1) thirst, hunger, and malnutrition, 2) discomfort and exposure, 3) pain, injury, and disease, 4) fear and distress, and 5) freedom to express normal behaviour (Webster, 1994; Webster, 2016). All of these freedoms relate to hedonic wellbeing, with the exception of freedom 5. Hedonic wellbeing is based upon pleasure and comfort seeking (Deci and Ryan, 2008). More recently, there has been a call in the literature and from the public for animals to have 'A Life Worth Living' (Mellor, 2016) or 'the Good Life' (Harfeld, 2013). As such, animal welfare concerns are moving away from merely ensuring that animals are provided with the opportunity to perform (by ensuring that they have adequate nutrition, freedom from fear, sickness, and discomfort), to ensuring that they have a life worth living (at least in terms of anthropomorphic understanding of 'worth'). This appeals to the eudaimonic theory of wellbeing. For further readings find Mellor (2016) and then the invited response, Webster (2016).

Under eudaimonic theory, wellbeing is a process and not a state. It stems from the pursuit of a good life through individual choices (Deci and Ryan, 2008). Much of what is known about eudaimonic wellbeing comes from philosophy, but recently scientific evidence has been gathered to support this theory using human subjects (Ryff, 2013). In grazing animals, more research is required to investigate eudaimonic wellbeing and we believe that experiments centred around providing choices are particularly needed. Evidence for support of Eudaimonia in livestock has been shown in zoo animals. Giant pandas had lower urinary cortisol when they were provided a choice between two environment enclosures compared to pandas who were only allowed access to the exhibit environment. As the added enclosure area was less enriched, the choice group spent most of their time in the exhibit area, and there were no differences in active time it was concluded that the enhanced animal welfare was derived from the ability of the animals to choose (Owen et al., 2005). In foraging ruminants, some support for telos and eudaimonic wellbeing may be seen in grazing personalities (Gregorini et al., 2017). One example of grazing personalities was described by Bailey et al. (2015), who found that there are cattle who prefer to graze in the flat low fields, termed bottom dwellers, and cattle who prefer to climb mountainous areas for grazing, termed hill climbers. It was found that these specific grazing personalities were related to genetic markers (Bailey et al., 2015). This is interesting as telos has been described as intrinsic in the genetic coding of animals (Harfeld, 2013). Thus, we hypothesize that individual animal's personalities, including grazing personalities, provide insight to individual animal's telos and thus provide evidence of eudaimonic wellbeing. Additionally, we speculate that this theory will apply to livestock and that enhanced welfare from dietary diversity both facilitates and is evidence to support this theory, which is discussed further below, even though separating welfare enhancement of dietary diversity between hedonic and eudaimonic wellbeing is difficult.

2.8 Linking Dietary Diversity and Physiological Stress

Choice is a key concept in the eudaimonic theory of human wellbeing, with the overarching concept being to pursue a life of fulfilment of one's true nature, or telos, with choices being important in

this pursuit (Deci and Ryan, 2008). Eudaimonia often stands in contrast to the hedonic theory of well-being, which considers contentedness as the sum of positive and negative affective states, i.e. emotions (Deci and Ryan, 2008) and which has been a primary focus of studies of animal welfare (Nordenfelt, 2011). However, recently several works have explored the effect of choice on livestock welfare. Catanese et al. (2013) gave lambs either a choice of different foods contrasting in protein:energy ratios (diversity) or all of those foods provided in a total mixed ration (monotonous). It was found that when animals were allowed to choose, they had lower cortisol, than their counterparts (Catanese et al., 2013). Villalba et al. (2012) has also shown similar results in lambs. When lambs were offered a four-way choice between foods which were diverse in nutrient composition or in PSC, there was lower plasma cortisol concentrations compared to lambs who received a monotonous diet of all food options. Manteca et al. (2008) and Villalba et al. (2010) reviewed dietary choice as an important aspect of animal welfare and related it to animals being able to balance their own nutrients to meet individual requirements through nutritional wisdom, and also to balance intake of PSC so that they can experience their benefits (e.g. reduced gastrointestinal parasites) without experiencing toxicities, which all relate to hedonic wellbeing. Additionally, hedonic wellbeing is partly responsible for controlling feeding behaviour in ruminants (Ginane et al., 2015). While these are likely true, dietary diversity may also reduce stress merely by providing the animals a choice, if the Eudaimonic theory of wellbeing can be applied to livestock. Additionally, we postulate that dietary diversity likely enhances both hedonic and eudaimonic wellbeing (Figure 2.1), as it has been found that these two mental wellbeing states contribute to welfare in different and overlapping ways (Huta and Ryan, 2010).

As mentioned previously, dietary choice allows animals to consume PSC at amounts that provide benefits, while staying below the threshold at which negative effects occur. In one experiment, sheep faced either no challenge (received saline injection), an adrenocorticotropic hormone (ACTH) challenge only, or an ACTH challenge plus one of four PSC (containing polyphenols) products (Sgorlon et al., 2012). Sgorlon et al. (2012) examined global mRNA expressions in sheep blood in response to the blood cortisol

levels, which resulted from the ACTH challenge. As expected, ACTH treatment caused increased cortisol production after 3 and 51 hours. While the sheep that received plant secondary compounds did not experience reductions in cortisol production, it was determined that the PSC altered the molecular signature produced as a result to increased cortisol. Overall it was determined that while ACTH challenge reduced gene expression involved in immune response, when provided PSC products, this effect was attenuated, but the results were dependent upon the product used (Sgorlon et al., 2012). Thus, PSC may improve the response that animals have to physiological stressful events.

There is a known relationship between diet and the composition of ruminal microorganisms. For example, Tapio et al. (2017), in a 4 x 4 factorial, fed dairy cows two levels of forage-to-concentrate (high, 35:65; low, 65:35) with either 0 or 50 g sunflower oil/kg diet dry matter. It was determined that there were taxa abundance changes and microbial interactions that were diet specific. Similar results have been seen in cows fed alfalfa or triticale forages (Kong et al., 2010). The composition of ruminal microorganisms can likewise influence the ruminal degradability of feeds, but also fermentation end products as specific microorganisms produce different fermentation end products, as they often fill dietary niches (Baldwin and Allison, 1983; Russell and Rychlik, 2001). As the majority of energy available for ruminants to use for metabolism come from fermentation end products (approximately 70% in ruminants; Bergman, 1990) and the ruminal microbiome composition determines the types of fermentation end products produced, several experiments have shown a link between the rumen microbiome composition and feed efficiency traits (McCann et al., 2014; Myer et al., 2015; Li and Guan, 2017). Therefore, the ruminal microbiome is an important aspect of ruminant nutrition and is dependent on diet.

Relatively recently, there has been much work on how microbial fermentation products (e.g. volatile fatty acids) can alter mood, behavior, and subsequently physiological stress, mental wellbeing, and welfare, through what is termed the microbiota-gut-brain axis. Much of this work has been done with humans. In a review, it was concluded that the gut microbiota in humans can communicate with the

central nervous system, subsequently altering mood, cognition, and emotions (Cryan and Dinan, 2012). Likewise, the microbiota-gut-brain axis has been shown to influence behavior (anxiety and social) and memory capacities in non-ruminant livestock (Kraimi et al., 2019). Microbial fermentation products are known to alter feeding behavior in ruminants and to provide positive postingestive feedback to the animals, thereby providing positive emotions and influencing the preference for specific foods. For instance, when different flavors were offered to sheep and associated with a low or high addition of exogenous propionate (a glucogenic volatile fatty acid), it was found that at lower additions ruminants developed a dietary preference for the conditioned flavor, whereas at the higher addition the sheep developed aversions to that flavor (Ralphs et al., 1995; Villalba and Provenza, 1997). This relationship makes intuitive sense as the volatile fatty acids provide 70% of the caloric requirements of ruminants (Bergman, 1990). In humans and other mammals, the microbiota-gut-brain axis has been shown to influence behavior, mood, and emotions, while the only predominant link shown in ruminants is its effect on feeding behavior (Kraimi et al., 2019). The availability of information on the microbiome-gut-brain axis and its effect on non-eating related behaviors in ruminants may be lacking because fermentation products represents a much larger contribution to their nutritional requirements than humans or other farm animals. For example, while volatile fatty acids contribute 70% of energy for metabolism in ruminants, it only accounts for approximately 10% for humans, 25% for pigs, and 30% for rabbits and horses (Bergman, 1990). However, there is still a need to determine how the ruminal and hind-gut microbiome may alter non-eating related behaviors in ruminants.

Dietary diversity would likely influence the ruminal microbiome composition, which in turn may influence the host animal's mood, emotions, and welfare and would influence dietary preference and dry matter intake. A review of the human literature concluded that a diverse diet would supply a wide range of substrates for the microbes to ferment in the gut, which would promote a more diverse microbiome (i.e. microbial species richness). This diverse gut microbiome was suggested to be more adaptable to disruption (Heiman and Greenway, 2016). It is known that diet formulation alters the

ruminal microbial species richness. For instance, when grain based diets are fed to ruminants they have a less diverse microbiome compared to forage based diets (Khafipour et al., 2016) and differences have also been shown when cows were fed different forages (Kong et al., 2010). However, to our knowledge, there is no information available for how dietary diversity may influence the ruminal microbiome's species richness. Therefore, there is a need to determine how dietary diversity may influence the microbiome of grazing ruminants. Additionally, while it is known that microbial fermentation products can alter dietary preference and intake behavior in ruminants, there is a lack of knowledge on if the fermentation products could influence other behaviors, and subsequently mental wellbeing and welfare, in ruminants. However, based on experimentation with other mammals (humans, rats, etc.), there is a strong possibility for dietary diversity to alter the microbiome, mood, and emotions when provided to ruminants.

Grazing ruminant mental wellbeing and nutrition are closely linked. Hedonic wellbeing influences voluntary feed intake through changes in opioid, cannabinoid, and the GABA systems, thus providing a reward response and influencing how ruminants like a specific food (Ginane et al., 2015; Figure 2.1). By providing dietary diversity, animal wellbeing may be improved in several ways. One is through improved eudaimonic wellbeing, by providing the animals with choice, thus allowing control over the animal's environment and the expression of individuality. Another means is through improved hedonic wellbeing by enhanced internal state by improved nutritional status. Additionally, PSC consumed at an appropriate level, which is allowed by dietary choice, have direct effects on the response that the animal has to physiological stress. Finally, dietary diversity may alter the microbiome-gut-brain axis, which has been shown to alter the mental wellbeing of other mammals.

2.9 Nutrition as Affected by Dietary Diversity

Ruminants have evolved in ecosystems where dietary choices abound and where they were able to select plants differing in PPC and PSC so that they could consume a balanced diet that met their needs for nutrients, medicine, and prophylactics (Provenza, 1996). Dietary diversity and allowing animals to

choose from an arrangement of feedstuffs to meet their own requirements is not a new concept (Provenza, 1996). As ruminant nutritionists, requirements are typically assessed and food offered to meet those requirements for an average animal. However, if we expect dietary requirements to follow a normal distribution, a small number of animals would be "average" and thus, approximately 50% of animals will be fed diets that under supply nutrients and around 50% will over ingest nutrients (Scott and Provenza, 1999; Atwood et al., 2001). Therefore, lack of dietary choice may result in individual dietary imbalances. These nutrient imbalances may lead to incidental restriction or augmentation (Villalba et al., 2015b). Incidental restriction is a reduction in intake due to negative post-ingestive feedbacks as a result of over consuming specific nutrients and incidental augmentation occurs when animals over ingest nutrients in order to meet their requirements for nutrients that are in lower concentrations in the diet (Villalba et al., 2015b). The differences between individual animals are a result of variations in physiological and morphological differences (Scott and Provenza, 1999) and also due to individual personalities (Meagher et al., 2017). In a grazing context, Parsons et al. (1994) found that, overall, sheep prefer to mix their diet and that their dietary preferences change across the day, influenced by sward characteristics and their previous diet. Parsons et al. (1994) measured preference by video-recording grazing location (i.e. forage species) and calculating forage intake from previously established intake rates of the respective forages. Individual animals vary greatly with regard to selection of dietary components within and between meals. It's because of these differences of individual animal preference and selectivity that common management goals aim to reduce sorting and selectivity by cattle fed "total-mixed rations" (Shaver, 2002). These management goals often involve adding liquids (e.g. molasses or water) to the mixed rations (Shaver, 2002). Interestingly several works have shown these management strategies actually encourage feed sorting and reduce dry matter intake and this has been related to the lower dry matter diets having greater temperatures resulting in increased spoilage (Miller-Cushon and Devries, 2009; Felton and DeVries, 2010).

The difference in individual animal selection is likely due to individual variation in the internal-state and post-ingestive feedback mechanisms that govern intake. This means that providing animals choice in the dietary constituents, rather than offered as a "total-mixed ration" formulated for the average animal or a non-functional mixed sward (mixed swards planted in a way that inhibit selection) may allow animals to choose from the dietary constituents in order to meet their respective requirements (Gregorini et al., 2017). Ruminant producers offering livestock high concentrate diets prefer to feed total mixed rations for ease of management and to reduce risks for negative health problems (e.g. ruminal acidosis and laminitis). However, it has been suggested that by offering choice ruminants can alter eating patterns to account for the later concern (Atwood et al., 2001) and this has been supported by experiments where grain was offered at free choice and pH was measured (Moya et al., 2011; Moya et al., 2014). While there has been much research on feeding total mixed rations in the last 60 years, (see Schingoethe, 2017), there is surprisingly few experiments which have compared total mixed rations compared to the dietary constituents offered as choice, but many of those that have, found choice to be superior. In an early experiment, reduced dry matter intake, similar performance, and improved feed efficiency were observed when dairy cows were offered forage and grain separately as opposed to being provided a total mixed ration (Nocek et al., 1986). Another experiment conducted in feedlot fed steers provides further evidence for this hypothesis (Atwood et al., 2001). Cattle were offered either a total-mixed ration or the components of the total-mixed ration offered individually. It was found that the diet selected by cattle varied tremendously between animals, but also within animals across days. The cattle offered choice consumed less feed, had similar performance, and lower cost of gain compared to the total mixed ration treatment (Atwood et al., 2001). A separate experiment conducted by the same laboratory with growing sheep found that when lambs were provided choice between three iso-caloric and iso-nitrogenous diets, they had greater dry matter intakes, performance, and feed efficiency, and less cost-of-gain compared to lambs offered only one of the three diets (Atwood et al., 2006). These experiments have been corroborated by other laboratories. When lactating goats

were offered choice they consistently consumed less dry matter comparable milk productions compared to their total mixed ration counterparts (Yurtseven and Görgülü, 2004). It is important to note that some experiments have found choice and total-mixed rations to be not significantly different (Moya et al., 2011; Moya et al., 2014) or for total-mixed rations to be superior (McCoy et al., 1966). Likewise, others contend that ruminants possess poor internal wisdom and that they are unable to select diets according to their nutrient requirements (Schingoethe, 2017). These different findings and conclusions may be due to the differences in the dietary options provided. If dietary constituents are not divergent enough in nutritive composition, then animals may not be able to select diets tailored to their specific individual nutrient requirements. Several experiments across multiple species and production settings have shown choice to improve feed efficiencies (either by reducing intake while maintaining intake or by increasing intake and performance) compared to offering a total-mixed ration, which were formulated to be optimal for the average animal. This is clear evidence for the importance of dietary choice as a means for meeting the individual requirements and avoiding incidental restriction or augmentation of intake.

2.10 Linking Oxidative and Physiological Stress

In humans, a link between oxidative status (metabolic stress) and physiological stress has been suggested and reviewed, with an apparent vicious cycle where physiological stress increases metabolic stress, which in turn increases physiological stress, etc., resulting in telomere shortening and aging (Ando and Fujita, 2009; Epel, 2009; Hopps et al., 2010). This may be especially true in scenarios of chronic stress (Hopps et al., 2010; Aschbacher et al., 2013). Aschbacher et al. (2013) explored the effect of chronic stress and perceived acute stress and found that there was significant oxidative damage when chronically stressed people experienced a perceived stressor. Chronic stress occurs when there are relatively high levels of glucocorticoids circulation in the blood stream for a prolonged period of time. Chronic stress has been linked to health problems in humans and animals. Several works have reported increased oxidative stress as a possible mode of action behind the cost of chronic stress (Costantini et al., 2011). Orzechowski et al. (2000) explored how rat's antioxidant status and oxidative stress changed

when challenged with dexamethasone (a synthetic GC; 2-mg/kg of body weight/d). It was found that treatment with dexamethasone decreased blood and muscle glutathione, reduced SOD activity, and increased malondialdehyde (measured by TBARS; Orzechowski et al., 2000). A meta-analysis by Costantini et al. (2011) concluded that GC were significantly associated with oxidative stress and that there were different magnitudes of effects according to tissue, sex, and age. Therefore, physiological stress increases oxidative stress in livestock and other mammals.

There is little direct evidence to link physiological stress and oxidative stress and their subsequent consequence in livestock. The experiments that have explored these relationships generally compared animals before and after a physiologically stressful event. In one experiment, 105 crossbred steers where transported for 19 hr and 40 min. This stressful event significantly reduced serum antioxidant capacity and increased malondialdehyde (marker of oxidative stress). It was found that calves with more incidence of bovine respiratory disease also had higher oxidative stress after transportation (Chirase et al., 2004). Other common management practices, which are known to be stressful to animals have been linked to oxidative stress. After sheep were shorn, there were greater circulating malondialdehyde (marker of lipid peroxidation) concentrations than before shearing (Fidan et al., 2009). Finally, malondialdehyde was likewise increased after cattle were dehorned (Fidan et al., 2010). These experiments provide evidence that physiological stress increases oxidative stress in livestock. However, there is less evidence to show that dietary antioxidants can reduce physiological stress. One experiment challenged sheep with injections of ACTH and found that a treatment group provided with supranutritional antioxidants (Vitamin E and Se) had lower circulating cortisol compared to their non-supplemented cohorts (Chauhan et al., 2014). Some recent works have shown a positive correlation between isoprostanes, which results from oxidant conversion of arachidonic acid, and cortisol. These experiments explored the effects of a nonsteroidal anti-inflammatory drug on reproductive performance of cattle (Kasimanickam et al., 2018; Kasimanickam et al., 2019). As mentioned previously, isoprostanes result from the peroxidation of arachidonic acid by oxidants and it

has been suggested that they are the pathophysiological mediators of oxidative damage (Montuschi et al., 2007). A positive correlation between cortisol and isoprostanes provides direct evidence and a potential mode of action for a link between oxidants and physiological stress. However, the relationship between physiological and oxidative stress is an area that requires further investigation in livestock, but there is evidence that improving oxidative status may allow the animal to better recuperate from the stress and reduce subsequent negative effects.

2.11 Linking Oxidative and Nutritional Status

Metabolic disorders seen in transition dairy cows provides excellent insight into how oxidative stress can be effected by the nutritional status of the animal. Bernabucci et al. (2005) followed 24 cows with different body condition scores (BCS) across the transition period (± 30 d at calving). It was concluded in this experiment that oxidative status was related to energy status and that cows with greater weight loss over this period experienced greater oxidative stress (Bernabucci et al., 2005). This positive relationship between energy demand and negative energy balance and oxidative stress has been shown in several experiments. For example, milk yield is positively correlated to markers of oxidative stress in dairy cows (malondialdehyde, Gabai et al., 2004 and hydroperoxides, Lohrke et al., 2004; Castillo et al., 2003). While most of the experiments focused on nutrition and oxidative stress in dairy cows, the relationships are applicable across ruminant species. For example, when lambs were fed 70% or 80% of their metabolizable energy requirements, they had higher plasma malondialdehyde levels than when lambs were fed 100% of their requirements (Singh et al., 2011). Level of energy intake is not the only way oxidative status is influenced by nutrition, in fact source of energy can have impacts. When lambs were fed high fat, there was an increase in blood superoxide dismutase levels and glutathione concentrations. This was attributed to increased fatty acid oxidation which would stimulate the production of oxidants (Sgorlon et al., 2008). Nutritionally related disorders such as subacute ruminal acidosis can also induce oxidative stress. Guo et al. (2013) found that when dairy cows were induced with subacute ruminal acidosis, there were lower plasma levels of total antioxidant capacity and higher

glutathione peroxidase activity and malondialdehyde concentrations. Nutritional status and oxidative stress are intimately linked. Oxidative stress can be influenced by previous level of nutrition, current energy intake, source of energy, and also nutritional related diseases.

As mentioned above, diet can greatly influence the composition of the ruminal microbiota. Further, we discussed above how microbial fermentation products can influence physiological stress and welfare. As physiological stress and reduced welfare will likely lead to oxidative stress, the effect that dietary diversity may have on the ruminal microbiota, or even the hind-gut microbiota, may provide another mode of action for dietary diversity to reduce physiological and oxidative stress. However, this requires further investigation. Additionally, microorganisms have been directly linked to reductions in oxidative stress. For instance, cattle had less antioxidant activity in their ruminal fluid and in their plasma when they were defaunated (removal of protozoa) compared to their faunated cohorts (Gazi et al., 2007). Also, steers placed in a feedlot had less glutathione peroxidase activity (indicating less oxidative stress) and a greater blood antioxidant level when provided a lactobacillus fermentation product compared to the control steers (Ran et al., 2019). Thus, dietary diversity may alter mood and behaviour, thereby influencing mental wellbeing and welfare, indirectly by altering the ruminal microbiota composition and also may directly reduce oxidative stress.

2.12 Concluding Remarks

This review has covered oxidative stress, physiological stress, and nutritional status, which are areas of animal science that are important to both producers and consumers. Further, we have provided links between these three areas and have described how dietary diversity links the three. In conclusion, there is evidence to support how dietary biochemical diversity (provided through taxonomical diversity) can reduce oxidative stress directly by providing plant secondary compounds as natural dietary antioxidants and indirectly by reducing physiological stress, which we have reported evidence to influence oxidative status. Additionally, the antioxidant benefits of plant secondary compounds may improve the metabolic response the animal has to physiological stress and therefore improve the

response to the perceived stress. Dietary diversity may improve eudaimonic wellbeing merely by allowing animals to make choices, and thus we postulate that this theory of wellbeing applies to livestock. Further, diverse diets may alter the microbial-gut-brain axis, which in humans and some non-ruminant farm animals has been shown to alter cognition, mood, emotions, and behaviour, as well as dietary preference and eating behaviour in ruminants. Finally, dietary choice allows animals to take advantage of differences in plant primary compounds to meet individual animal requirements and thereby improve nutritional status. Improved nutritional status can subsequently have beneficial impacts on oxidative stress by reducing energy store mobilization and physiological stress by improving hedonic wellbeing. Physiological stress, oxidative stress, and nutritional stress are intimately linked (Figure 2.1) and dietary choice compared to monotony may simultaneously improve all of these items directly and indirectly, resulting in marked improvements in the foraging animal's nutritional status, health, mental wellbeing, and ultimately their welfare. We conclude that dietary diversity reduces stresses while enhancing hedonic and eudaimonic wellbeing in ruminant livestock.

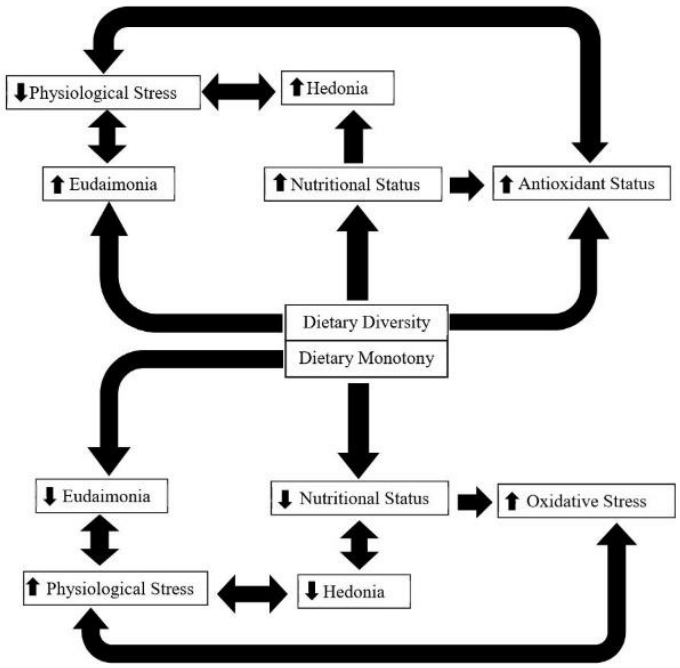

Figure 2.1 This figure shows a conceptual model depicting how dietary diversity vs monotony may affect ruminants. Dietary diversity improves nutritional status, through ingesting complimentary plant compounds (primary and secondary); improves hedonic and eudaimonic wellbeing; decreases physiological stress; and improves antioxidant status, through the ingestion of plant secondary compounds that exhibit antioxidant effects. It is important to note that both nutritional status and physiological stress can also impact antioxidant status directly. On the other hand, dietary monotony may reduce nutritional status (by not allowing animals to mix a diet containing a balance of plant primary compounds) and hedonic (reduced nutritional status) and eudaimonic (loss of choice) wellbeing.

2.13 References

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Chapter 3

Low levels of a seaweed (*Ecklonia radiata*) extract alters *in vitro* fermentation products but not in combination with quebracho (*Schinopsis quebrachocolorado*) tannins.

3.1 Abstract

The objective of this experiment was to determine the effect of increasing doses of a seaweed extract (*Ecklonia radiata*; SWE) and SWE with quebracho (*Schinopsis quebrachocolorado*) tannin extract (TE), on *in vitro* fermentation. Two *in vitro* experiments were conducted using the Daisy II incubator, where repeated measurements were taken. In experiment 1 (Exp1), treatments were increasing levels (0, 2, 9.5, or 31.5-µl/fermentation jar) of the SWE and in experiment 2 (Exp2) treatments were either no SWE or TE (CON), the optimal dose (the regression minimum) determined from Exp1 of SWE only (SWO), 4-mg TE and SWO (LTS), or 10-mg TE and SWO (HTS). There were no time-by-dose interactions ($P \geq 0.13$) in either experiments. Only the NH_3 from the 2-µl dose was different ($P = 0.04$) from the 0-µl dose. No other differences ($P > 0.10$) were detected in Exp1. A mixed model regression determined a cubic relationship between dose and NH_3 ($P = 0.02$), with the minimum at 5-µl. In Exp2, SWO reduced ($P = 0.02$) NH_3 compared to all other treatments. The SWO treatment also reduced total VFA compared to the other treatments ($P = 0.01$). The LTS and HTS were not different from the CON for any variables ($P > 0.1$). Under the context of this experiment, we conclude that low levels of SWE reduce ruminal NH_3 and suggest that urinary N may be reduced *in vivo*, but was negated in the presence of condensed tannins.

Keywords: *in vitro*, seaweed, rumen fermentation, tannins

3.2 Introductions

A consequence of overfeeding nitrogen (N) to ruminants is excess rumen ammonia (NH_3) production and subsequently increased amounts of urinary N excretion. The N deposited through urine is at a rate too high for sward plants to utilize. Excess urinary N is lost from the system through N leaching, NH_3 volatilization, or N_2O emissions, all of which have negative environmental implications (Gardiner et al., 2016). Altering volatile fatty acid (VFA) production by reducing non-glucogenic fermentation is beneficial as it can reduce methane (CH_4) emissions (Johnson and Johnson, 1995) and improve animal performance (Bergen and Bates, 1984). Seaweeds have shown the potential to alter fermentation parameters (ruminal-NH_3 and VFA profiles) *in vitro* (Machado et al., 2014; Hong et al., 2015; Maia et al., 2016) and *in vivo* (Li et al., 2016); however, type of seaweed appears to influence these effects (Machado et al., 2014; Maia et al., 2016). The observed benefits of seaweed have been related to their plant secondary compounds (PSC; phlorotannins and essential oils; Gupta and Abu-Ghannam, 2011). Additionally, tannins have been shown to alter fermentation patterns and reduce ruminal NH_3 and CH_4 production, thereby reducing environmental impacts of livestock (Waghorn, 2008). A commercial product is available in New Zealand that is based on the fermentation extract of a brown seaweed (*Ecklonia radiata*; SWE, Agrisea Ltd, Paeroa, New Zealand). This product is marketed to improve animal production; however, there is currently no information available on the appropriate dose of SWE or any information on how this product can be influenced by the addition of tannins. We hypothesized that SWE would alter ruminal fermentation patterns by shifting VFA production and reducing NH_3 and that these results would be improved by adding tannins. The objective of this study was to determine the appropriate dose of SWE, and the potential improvement of the determined SWE dose with the addition of quebracho (*Schinopsis quebrachocolorado*) tannins.

3.3 Materials and Methods

All procedures used in this study have been approved by the Lincoln University Animal Ethics Committee (AEC 2018-08).

3.3.1 Seaweed Extract Analysis

Total phenolics of the SWE was determined using the Folin-Ciocalteu reagent and gallic acid as a standard. Two 1-mL samples of SWE were aliquoted and centrifuged at 10,000 rpm for 5 min. The supernatant was taken off and combined before being filtered through a 0.45 µ PTFE filter. The filtrate was separated into a 5 and 10 times dilution. Folin-Ciocalteu reagent were added and absorbance was recorded at 765 nm. The absorbance was then placed into the calibration equation (R^2 = 0.99) to calculate total phenolic content in mg of gallic acid equivalence per ml of SWE (0.83 mg of GAE/mL). Further description of the seaweed extract chemical and nutritional composition can be found at the Agrisea website (https://agrisea.co.nz/industries/dairy/animal-nutrition/).

3.3.2 Animal Handling, in vitro fermentation, and treatments

Non-lactating, non-pregnant rumen cannulated cows (Holstein and Jersey cross; n = 4; BW = 700 kg) were housed at the Lincoln University Johnson Memorial Lab Research Farm and were used for collection of ruminal fluid. Cows were grazed on a predominantly perennial ryegrass (*Lolium perenne*) diet. All methods used followed those described by Al-Marashdeh et al. (2017). In brief, two of the 4 cannulated cows were fasted overnight and the 4 cows were rotated between each sampling period so that each animal was only used twice for these experiments (4 fermentation runs per experiment). Fluid collection consisted of grabbing digesta from random parts of the rumen from both fasted cows and squeezing the fluid from the digesta into two pre-warmed (39.5°C) 1-L thermoses. Ruminal fluid from the two cows was brought back to the lab, strained through cheesecloth, composited by a blender, and 400-mL of the composited fluid were measured into four commercially available fermentation jars, which contained a buffer that was prepared according to the incubator operators manual (ANKOM, 2013). Throughout the fluid preparation, care was taken to maintain an anaerobic environment by purging the ruminal fluid with CO_2. After the ruminal fluid was added to the fermentation jars, 6.25 g of a perennial ryegrass substrate (described below), treatments were applied, and incubated for 24 h. Treatments were randomized to jar location during each run.

One kg of fresh perennial ryegrass (*Lolium perenne*) herbage was harvested on the 16 (Exp1) and 27 (Exp2) of February 2018, from Lincoln University Research Dairy farm and stored at -20°C. Harvest consisted of hand clipping forage to 3-cm above ground level. Samples were then freeze-dried and ground to pass through a 1-mm screen using a centrifugal mill (ZM200 Retsch).

For experiment one (Exp1), treatments were the addition of: 0, 2, 9.5, or 31.5 µl of SWE. These treatments equate to 0, 0.32, 1.52, or 5.04 µl per g of forage. Assuming a daily DMI of 17 kg/d for a grazing dairy cow, these levels would be equivalent to provide, 0, 5, 25, and 80 mL per head per d.

The optimal dose (amount of SWE to add for the best results based on the reduction of NH_3) determined in experiment one was used for the design of treatments in experiment 2 (Exp2), which consisted of either: no SWE and no tannins (CON; Silvafeed ByPro; San Michele Mondovi, Cuneo, Italy), optimal dose of SWE only (SWO), low tannins (4 mg) and optimal dose of SWE (LTS), or a greater amount of tannins (10 mg) and the optimal dose of SWE (HTS). The low and high levels of tannin additions would be similar to providing a dairy cow, consuming 17 kg/d, 0.06%, and 0.2% of their diet, respectively. These incorporations of tannins are lower than other experiments. Hervás et al. (2003) found that quebracho tannins incorporated at 17% of dietary DM was toxic to rumen microbes, 8% of dietary DM significantly reduced ruminal fermentation, and 3% of dietary DM showed no adverse effects. However, at similar inclusions (0.2% dietary DM) to the current experiment, a shift in microbial populations and a reduction in urease activity have been observed *in vivo* (Carrasco et al., 2017). Additionally, the intention of the tannins was not to provide levels which are typically used when tannins are fed alone, but to see if small additions might synergistically benefit the SWE.

3.3.3 Samples Collection and Laboratory Analysis

Samples of ruminal fluid were taken for each run at 0, 2, 4, 6, 8, 12, and 24 h after incubation began. Two, 2-mL samples were placed into either a 2-mL Eppendorf tube with 10 µl of sulfuric acid for NH_3 analysis, or one without acid for VFA analysis. Ruminal NH_3 analysis was conducted by an enzymatic UV method (Neeley and Phillipson, 1988) using a Randox Rx Daytona (Crumlin, County Antrim, UK).

Volatile fatty acid analysis was conducted using a gas chromatograph (Chen and Lifschitz, 1989; Shimadzu GC-2010; Kyoto, Japan). Herbage samples were analysed for chemical composition using near infrared spectroscopy (Corson et al., 1999) and chemical composition is provided in Table 3.1. A subsample of the herbage was oven-dried at 60°C to determine DM content.

3.3.4 Statistical Analysis

The experimental unit used for both experiments was fermentation jar (n = 4 per run) in each incubation (n = 4 per experiment). A randomized complete block design was used for both Exp1 and Exp2. A repeated measures mixed model ANOVA was fit by REML, with treatment, hour of sampling, and their interactions as fixed effects. Error of sampling time within run was the random effect. After a significant ANOVA, means seperations were done by a pairwise t-test using the 'emmeans' package (Lenth, 2018) of R (R Core Team, 2018, v. 3.4.4.). Additionally, a linear mixed model was fit for some of the dependent variables (experiment one only). Final model selection was dependent on the reduction of Bayesian Information Criterion (BIC). This was not the primary means of statistical analysis for experiment one due to the limitation of the number of independent observations (dose = 4 levels) and the potential of overfitting during this experiment (Babyak, 2004). Random intercept effects were selected to account for the differences observed between sampling hours within runs and included hour nested within run. Statistical significance was declared at $P = 0.05$ and trend was $0.05 < P \leq 0.1$. All statistical analysis was conducted using R (R Core Team, 2018, v. 3.4.4.).

3.4 Results and Discussion

There was no treatment by time interactions in any of the two experiments so only the main effect of treatment is reported.

3.4.1 Experiment 1

Dose of SWE did not affect pH ($P = 0.37$; Table 3.2). Dose affected rumen NH_3 concentration ($P = 0.04$). The 2-µl dose reduced ($P \leq 0.03$) NH_3 concentration compared to the 0 and 31.5 µl doses, but was not smaller than the 9.5 µl dose ($P = 0.35$). The 9.5 µl dose tended to produce less ($P = 0.1$) NH_3

concentration than the 0 µl dose, and was not significantly different (P = 0.23) from the 31.5 µl dose. The 0 and 31.5 µl doses did not differ (P = 0.65; Table 3.2). In order to determine the dose used in Exp2, a mixed model regression was fit (Table 3.3). A cubic relationship was observed (P = 0.02) with the local minimum at 5 µl of SWE. This dose was used in Exp2.

There was a trend (P = 0.08) for a dose effect on acetate concentration with 9.5 µl being smaller than the other treatment levels (Table 3.2). Dose did not affect propionate (P = 0.13), butyrate (P = 0.31), iso-butyrate (P = 0.21), valerate (P = 0.57), or iso-valerate concentrations (P = 0.89; Table 3.2). There was a trend (P = 0.08) for a dose effect on acetate to propionate ratio, with the 9.5 µl dose being reduced compared with the other treatments (Table 3.2). When fit with a mixed model regression, there was a trend (P = 0.1) for a negative linear relationship between dose and propionate and butyrate concentrations (Table 3.3). Experiment 1 indicates that only the lowest level of SWE reduced rumen NH_3 (5% reduction) concentration *in vitro*. As each fermentation jar contained the same volume, concentration in this case would be equivalent to total pool.

3.4.2 Experiment 2

The LTS treatment had a greater (P < 0.01) pH than the other treatments, but this was a minor effect as LTS was only 0.01 pH greater than CON and is likely not biologically significant. No other treatments were different (P ≥ 0.34) from each other in pH. The SWO treatment reduced (P = 0.02) NH_3 concentration compared with all other treatments, while no other treatments were different (P = 0.47) from CON. As in Exp1, sampling time affected (P < 0.01) all individual VFA, total VFA concentration, and the acetate to propionate ratio (Table 3.4). Treatment did not affect acetate to propionate ratio, butyrate, or iso-butyrate (P ≥ 0.11). However, SWO reduced (P ≤ 0.05) total VFA, acetate, valerate, and iso-valerate concentrations, compared with the other treatments. Other differences were for propionate concentration, with SWO having the lowest (P ≤ 0.04), HTS as intermediate and smaller (P = 0.04) than CON, but similar (P = 0.45) to LTS. The LTS treatment did not differ from CON (P = 0.18). Similar to Exp1,

the SWO treatment reduced rumen NH_3 concentration compared to the other treatments and this effect was lost when both levels of tannins were incorporated.

There were no effects on VFA by SWE in Exp1, but SWO significantly reduced total VFA, acetate, propionate, valerate, and iso-valerate in Exp2, which would indicate negative effects on fermentation. These effects may be due to the larger dose of SWE used in Exp2 than in Exp1. However, the 9.5 and 31.5-µl treatments used in Exp1 also did not reduce VFA concentrations. Thus, this effect is difficult to explain, but would indicate a dose dependent effect of the SWE, just as there is a dose dependent effect of SWE on NH_3.

Low additions (2 and 5 µl/incubation jar) of SWE reduced NH_3 production by 5-6% in Exp1 and Exp2 compared to the CON. Interestingly, when 5 µl of SWE was entered into the regression equation fitted by data from Exp1, there was a 6.8% reduction predicted. This was similar to the 5.6% reduction observed in Exp2 and these differences may be due to the CP differences of the substrates used. The reason why greater levels of SWE did not influence NH_3 production is unclear but would be dependent on the mode of action. If these reductions were due to the plant secondary compounds provided by the seaweed, namely phlorotannins, then it is possible that greater doses shifted bacteria populations so that more protein was utilized for energy (see Leng and Nolan, 1984 for a review on protein metabolism in the rumen), thereby releasing NH_3 at a similar level to the control. As the SWE is a fermented product, it is also possible that these results were due to probiotic effects. If this was the mode of action, the introduced bacteria may have had less affinity to utilize protein for energy. Different classes of bacteria have affinities for different substrates and will utilize these substrates based upon the concentrations of their preferred and the less preferred substrates (termed relative substrate affinities; Russell and Baldwin 1979). If this is the case then at low levels, the introduced bacteria did not have competition for their preferred substrate at a level where they needed to utilize protein and release NH_3 (Leng and Nolan, 1982). At the greater doses, the increased demand for the substrate that they prefer to use, because of the larger number of introduced bacteria, may have forced some of the introduced bacteria

to utilize protein for energy, thus producing NH_3 at a similar level of the no addition treatment (Leng and Nolan, 1982).

Adding quebracho tannins negated any effect observed by the SWO treatment (Table 3.4). Conversely, the SWE appears to have blocked any potential effect of the tannins, but this is difficult to conclude as we did not include a tannin only treatment. This indicates an interaction between the SWE and the tannin product. Tannins bind to different compounds, most notably proteins (Waghorn, 2008). It is possible for tannins to bind to other PSC, and if SWE mode of action was through PSC, a possible interaction may have masked the effects of SWE. In addition, tannins often have antimicrobial effects (Scalbert, 1991). The levels of tannins used in this study were likely not great enough to inhibit the ruminal bacteria (see Hervás et al., 2003) and that the tannins were more potent to the bacteria present in the SWE. We postulated, that if SWE provides probiotic effects that the bacteria introduced by SWE were inhibited by the tannins.

3.5 Applications

Our results suggest, that low levels of SWE additions reduce ruminal-NH_3 production by 5-6%. When extrapolated to mL of SWE per kg of DMI basis, this low level is similar to 5 mL per cow per d. We speculate a binary mode of action for SWE, being seaweed PSC and a probiotic effect from the bacteria fermenting the seaweed during the extraction process, but more evidence is required to support this. Finally, the addition of tannins negates the effects observed by SWE. More research is required on modes of action as well as to determine if this level of NH_3 reduction *in vitro* would result in biologically significant reductions *in vivo* to reduce urinary N excretion.

Table 3.1 Nutrient composition of the ryegrass (*Lolium perenne*) herbage used as substrate determined by near infrared spectroscopy (NIRS) used in experiment one and two.

Item[a]	Experiment one	Experiment two
rDM, % as-fed	95.7	94.7
NDF, %DM	46.0	46.6
ADF, %DM	26.9	29.8
CP, %DM	18.3	16.5
WSC, %DM	11.3	10.4

[a] rDM = residual dry matter; NDF = Neutral Detergent Fiber; ADF = Acid Detergent Fiber; CP = Crude Protein; WSC = Water Soluble Carbohydrates; %DM = percent dry matter basis.

Table 3.2 Experiment one least square means for fermentation parameters averaged across sample hours. Treatments were different doses of a seaweed extract.

	Dose, µl of SWE [x]					*P*-value		
Item	0	2	9.5	31.5	SE [y]	dose	time	Inter.
pH	7.03	7.04	7.03	7.04	0.03	0.37	0.03	0.99
NH_3, mmol/L	10.9[a]	10.4[b]	10.6[a,b]	10.8[a]	0.88	0.04	<0.01	0.84
Total VFA, mmol/L	17.2	17.1	17.4	17.6	2.55	0.65	<0.01	0.70
Acetate, mmol/L	11.6	11.5	11.7	11.8	1.77	0.82	<0.01	0.76
Propionate, mmol/L	3.0	3.0	3.1	3.1	0.44	0.13	<0.01	0.46
Butyrate, mmol/L	1.5	1.5	1.5	1.5	0.24	0.32	<0.01	0.47
Iso-Butyrate, mmol/L	0.3	0.3	0.3	0.3	0.06	0.21	<0.01	0.39
Valerate, mmol/L	0.2	0.2	0.2	0.2	0.05	0.57	<0.01	0.99
Iso-Valerate, mmol/L	0.5	0.5	0.5	0.6	0.09	0.89	<0.01	0.89
A:P [z], mmol/mmol	3.9	3.9	3.8	3.9	0.10	0.08	<0.01	0.99

[a-c] different superscripts differ ($P \leq 0.05$)

[x] SWE = Seaweed (*Ecklonia radiata*) extract (SWE, Agrisea Ltd, Paeroa, New Zealand)

[y] SE = Standard error of the mean

[z] A:P = Acetate to Propionate ratio

Table 3.3 Fixed effects coefficient, random effects, and fit parameters from mixed models fit by REML to data from *in vitro* experiment one. Dose of a seaweed (*Ecklonia radiata*) extract was the independent variable. These curves were fit only to estimate the optimal dose of the seaweed extract.

	Fixed Effects [a]								Random Effects Variance, % [b]		Fit Par.[c]
Item[d]	Inter.	SE	Linear	SE	Quad.	SE	Cubic	SE	Rand. Effect	Resid	BIC
NH_3	10.9***	0.9	-0.32***	0.12	0.039**	0.016	-0.001**	0.0004	87.3	12.7	441.0
C2	11.6***	1.8	0.01	0.01					85.5	14.5	466.8
C3	3.0***	0.4	0.004*	0.002					87.5	12.5	175.1
C4	1.5***	0.2	0.002*	0.001					87.5	12.5	10.5
C2:C3	3.9***	0.1	-0.002	0.001					71.1	28.9	73.3

[a] Coefficients and their standard errors of intercept, linear, quadratic and cubic terms of Dose

[b] Random effects are Run and between run error of sampling time

[c] Fit Parameter; BIC = Bayesian information criterion

[d] All units are in mmol/L except C2:C3 is mmol C2 per mmol C3; NH_3 = Ammonia, C2 = Acetate, C3 = Propionate, C4 = Butyrate, C2:C3 = Acetate-to-Propionate ratio

*, **, *** represent $P \leq 0.1$, 0.05, and 0.01, respectively, for the fixed effects.

Table 3.4 Least square means for fermentation parameters from experiment two. Treatments were a control (CON), the addition of a seaweed (*Ecklonia radiata*) extract (SWE) only (SWO), SWE plus 4-mg of quebracho (*Schinopsis quebrachocolorado*) tannins (LTS) or SWE plus 10-mg of quebracho tannins.

	Treatment [w]					*P*-value [x]		
Item	CON	SWO	LTS	HTS	SE [y]	Treat.	Hour	Inter.
pH	6.81[b]	6.80[b]	6.82[a]	6.81[b]	0.005	<0.01	0.11	0.98
NH_3, mmol/L	11.3[a]	10.6[b]	11.1[a]	11.1[a]	0.21	0.02	<0.01	0.26
Total VFA, mmol/L	15.0[a]	14.0[b]	14.9[a]	14.7[a]	1.02	<0.01	<0.01	0.75
Acetate, mmol/L	10.3[a]	9.6[b]	10.2[a]	10.0[a]	0.67	<0.01	<0.01	0.47
Propionate, mmol/L	2.4[a]	2.2[c]	2.3[ab]	2.3[b]	0.15	<0.01	<0.01	0.88
Butyrate, mmol/L	1.2	1.2	1.2	1.2	0.15	0.23	<0.01	0.98
Iso-Butyrate, mmol/L	0.4	0.4	0.4	0.4	0.02	0.11	<0.01	0.59
Valerate, mmol/L	0.2[a]	0.1[b]	0.2[a]	0.20[a]	0.04	0.01	<0.01	0.87
Iso-Valerate, mmol/L	0.6[a]	0.5[b]	0.6[a]	0.6[a]	0.03	<0.01	<0.01	0.83
A:P [z], mmol/mmol	4.4	4.4	4.5	4.4	0.09	0.68	<0.01	0.37

[a-c] different superscripts differ ($P \leq 0.05$)

[w] CON = no seaweed and no tannin, SWO = 5-µl of seaweed extract only, LTS = 4-mg of tannin product and 5-µl of seaweed extract, HTS = 10-mg of tannin product and 5-µl of seaweed extract.

[x] ANOVA *P*-values, Treat. = treatment main effect, Hour = Sampling time effect, and Inter. = interaction between treatment and sampling time.

[y] SE = Standard error of the mean

[z] A:P is acetate-to-propionate ratio and is mmol Acetate/mmol Propionate

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Chapter 4

Seaweed and bioactive rich terrestrial plant extracts enhance rumen fermentation and health of dairy cows during the last third of gestation and transition period.

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This chapter has been submitted to *Animals.*

4.1 Abstract

The objective of this experiment was to determine how commercially available fermented seaweed (Ecklonia radiata) (SWO; Animal Health Tonic; AgriSea Ltd.; Paeroa, NZ) and SWO plus a fermented extract of terrestrial plants (SWP; Aleviate; Agrisea Ltd.; Paeroa, NZ) products influences dairy cow rumen fermentation patterns and oxidative stress. Twenty-seven Holstein Friesian x Jersey dairy cows (18 non-cannulated and 9-cannulated) were randomly allocated to one of three treatments, 5-mL/d of either SWO, SWP, or water (CON), administered through a daily oral drench over 80 days in the last third of gestation and 100-mL/d of each treatment after calving (94 ± 5 days after trial initiation). Samples were taken on d 0 (prior to treatment application), d 38, d 80, and then 3-d post calving (i.e. d 97 ± 5). Cows receiving SWP tended to have a greater rumen concentration of acetate and butyrate ($P < 0.10$) and had a greater ($P \leq 0.04$) production of both propionate and valerate than the CON cows. In all cases of volatile fatty acid (VFA) concentrations, SWP was numerically greatest, SWO was intermediate, and CON was the least, resulting in SWP having a 26% increase ($P = 0.05$) of total VFA concentration compared with CON. Cows on CON had greater glutathione peroxidase (GPx) activity than cows on SWP and SWO at all-time points following treatment application, with the exception of cows on SWO having no difference ($P > 0.05$) in GPx activity to the other treatments following calving. After 80-d of applying

the treatments and 3-d post calving, SWO and SWP had greater total antioxidant status, indicating less oxidative stress compared with CON. Three days after calving, SWO and SWP had less ($P < 0.05$) non esterified fatty acid (NEFA) concentration in the blood than CON, indicating a reduction in lipolysis. Collectively, these results highlight the ability for these plant extracts to improve ruminal fermentation, increase BCS, and improve health and therefore wellbeing of dairy cows in the last trimester of pregnancy and across the transition period.

Key Words: Transition dairy cows, antioxidants, oxidative stress

4.2 Introduction

Pastoral dairy-production systems are under increasing social and consumer pressures to enhance animal health and welfare (Gregorini et al., 2017; Leroy, 2019). Such pressures are in part related to concerns on intensive and dietary-bland monotonous pastoral dairying on animal health and wellbeing (Manteca et al., 2008; Villalba et al., 2010; Gregorini et al., 2017). Animals consuming a monotonous forage [i.e. perennial ryegrass (*Lolium perenne*)] impairs the ability of animals to consume and self-medicate on plants containing beneficial plant secondary compounds, which can potentially impart anthelmintic and antioxidant benefits, to name a few (Villalba et al., 2019). This consequence of dietary biochemical monotony —typical of intensive pastoral systems— incurs increases of physiological and oxidative stress, which are related decreases in animal productivity, health, welfare, and wellbeing (Beck and Gregorini, 2020). Accordingly, it has become commonplace for research providing antioxidant supplements to grazing ruminants (e.g. plant extract) to increase antioxidant ingestion, which may otherwise be expected as a staple of their diet if provided a diverse arrangement of forages, in order for these animals to benefit from their medicinal properties (Abuelo et al., 2015; McGrath et al., 2018).

Physiological and metabolic stress are intimately associated and have been related to a mutual reinforcement cycle (Ando and Fujita, 2009; Beck and Gregorini, 2020). In dairy cows, stressful events such as pregnancy, calving and transition have been shown to increase oxidative stress, resulting from high

metabolic demand, and are a cause of common illnesses (i.e. mastitis, metritis, retained placenta, milk fever, etc.) (Lykkesfeldt and Svendsen, 2007). Moreover, increased morbidity and mortality as a result of physiological stress, has been related to reduced antioxidant status (Chirase et al., 2004). In searching for sources of livestock feed supplements with beneficial antioxidant effects, seaweed products have been reported to alter oxidative stress in livestock and alter rumen fermentation patterns in ways that improve health and reduce the negative energy balance of forage fed dairy cows. For example, a brown seaweed (*Ascophyllum nodosum*) extract (Tasco, Acadian Sealants Ltd, Nova Scotia, Canada) increased the antioxidant status in sheep challenged with heat stress (Saker et al., 2004) and cattle grazing endophyte infected tall-fescue (*Festuca arundinacea*) (Allen et al., 2001; Fike et al., 2001). Kannan et al. (Kannan et al., 2007) also reported antioxidant benefits of the brown seaweed extract when fed to goats challenged with transportation stress. Recently, several works have illustrated how low levels of red seaweed addition (0.10-1% of intake) has large impacts on enteric methane emissions, with concomitant production benefits (Roque et al., 2019; Kinley et al., 2020). The health and production benefits arising from feeding seaweed to cattle has been attributed to its chemical composition on the basis of bioactive components (Evans and Critchley, 2014; Beck and Gregorini, 2020). Furthermore, it is possible that increasing taxonomic and thereby biochemical bioactive diversity can improve the beneficial effects of plant extracts due to synergistic modes of action (Gregorini et al., 2017). Accordingly, biochemical diverse and enriched diets can enhance livestock, health and wellbeing (Beck and Gregorini, 2020).

We hypothesize that providing a seaweed-based fermented extract to late gestating dairy cows improves production, wellbeing, and health. We further hypothesize that increasing taxonomic and thereby biochemical bioactive diversity during the plant extraction process further enhance the beneficial results. The objectives of this experiment are to determine if and how seaweed and a seaweed plus terrestrial plant extract influence ruminal fermentation, and oxidative stress of dairy cows across non-lactating to lactation transition period.

4.3 Materials and methods

This experiment was conducted at the Lincoln University Research Dairy Farm from 7 May 2018 to 26 August 2018. All animal procedures conducted in this experiment were approved by the Lincoln University Animal Ethics Committee (AEC 2018-08).

4.3.1 Description of Products

The current experiment was designed to explore the health, in terms of ameliorating oxidative stress, and rumen fermentation effects of two *Lactobacillus* fermented plant products. One of the products is based on the fermentation of a brown seaweed (Ecklonia radiata) (SWO; Animal Health Tonic, AgriSea New Zealand Seaweed Ltd.; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand). The other product is based on the fermentation of seaweed, and other terrestrial plants (SWP; Fortress+; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand). Both extract products followed the extraction process, which was based on a fermentative extraction technique of the seaweed, in the case of SWO, as well as the seaweed and the terrestrial plants for SWP according to proprietary methods (Bradley; AgriSea New Zealand Seaweed Ltd.). These extracts are thought to provide health benefits through providing Lactobacillus fermentation metabolites of compounds extracted from the plant species, prebiotics, and probiotics. This was confirmed by assays which determined the SWO and SWP had 2,2-diphenyl-1-picryl-hyrazyl-hydrate (DPPH) inhibitions of 33.9% ± 1.4 and 48.7% ± 1.2 (mean ± standard deviation), respectively, which are similar to seaweed extracts reported previously (O'Sullivan *et al.* 2011). Additionally, these extracts contained *Lactobaccillus paracasei*. The methods used to determine these aspects of the products are described in greater detail in Chapter 6.

4.3.2 Animal Management and Feeding

Nine cannulated and 18 non-cannulated, pregnant non-lactating dairy cows (Holstein-Friesian × Jersey cross; 4.43 ± 8.16 BCS; 10 August 2018 ± 8 d expected calving date; Mean ± SD) were initially stratified by body condition score and expected calving date and randomly allocated within stratification to one of three treatments: 1) daily dosed with water (CON), 2) daily dosed with SWO, or 3) daily dosed SWP. Treatments were applied beginning on d 1 (8 May 2018), which was approximately the last 1/3 of gestation.

Prior to calving, cows received 5 mL/ d of their respective products, which were diluted with water so that each received a 20 mL oral drench daily. This dose corresponds to the SWO manufacturer's recommendations, verified by (Beck, Al-Marashdeh, *et al.* 2019). After calving, cows received (according to treatment) 100 mL/d for the remainder of the experiment. The dose was increased at this time as cows can experience a 20-fold decrease in energy balance after calving, resulting in large magnitudes of fat mobilization and oxidative stress (Grummer 1995).

Animals were maintained in a fallow paddock throughout the experiment. Cows were group-fed once daily at 0800, after application of their treatments, by unrolling (Chainless XL105; Hustler Equipment; Hastings, New Zealand) 1.5 bales (310 kg DM/bale, SD = 46.6 kg, n = 37) of lucerne (*Medicago sativa*) baleage. This offered approximately 17 kg DM/cow/d. Assuming 20% waste, forage allocation was estimated at 14 kg DM per cow per d. Baleage samples were analyzed by near infrared spectrophotometry (**NIRS**; Model: FOSS NIR System 5000, Maryland, USA). Feeding levels were designed to be sufficient for cows to gain 0.5 BCS (Scale 1-10) (Roche *et al.* 2004) by calving. Body condition scores were determined for each cow fortnightly for the duration of the experiment by a certified assessor (Dairy New Zealand 2018).

Fourteen ± 5 days prior to calving, cows were removed from the grass-free enclosure and placed into perennial ryegrass-based (*Lolium perenne* L.) swards. Cows were offered a daily herbage allowance of 16 kg DM/cow/d above 3 cm sward height for non-lactating cows and then 20 kg DM/cow/d above 3

cm following calving. The area provided to the cows was allocated using temporary electric fencing with the area required to meet the target forage allowances calculated on the basis of pre-grazing herbage mass, using a plate meter with established calibration equations (Jenquip; Feilding, New Zealand). Cows were maintained in a non-lactating herd until calving, and then, moved to a colostrum herd for 5 days. After the colostrum phase, cows were removed from the current experiment to a milking herd. Herbage samples from the allocated herbage were collected by clipping herbage in a 0.10-m^2 area in 3-random locations three times during this phase of the experiment. Feeding management was done to ensure that the metabolizable energy (ME) requirement of 130 MJ of ME per day for maintenance, gestation, and body weight change were met during the transition period (Nicol and Brookes 2007).

Cows were managed to utilize an automated head chamber system (AHCS) during both the dry and lactation periods of the experiment (Gunter and Beck 2018). This system was used to measure the gas emissions of the livestock, which will be reported in a subsequent publication. However, as the system uses a pelleted feed to entice the animals to visit and have their emissions measured, the intake of the pelleted feed is reported in this manuscript.

4.3.3 Animal sampling

All sampling was performed on d 0 (7 May 2018), d 38 (14 June 2018), and d 80 (26 July 2018), and 3-d after calving (blood sample only). Rumen fluid was obtained from rumen cannulated cows only (n = 3 per treatment) by collecting digesta through the cannula and squeezing the fluid through 2 layers of cheese cloth. A subsample of the collected fluid was placed into two, 2-mL Eppendorf tubes, one acidified with 10-µL of 98% sulfuric acid (Fisher Scientific, Loughborough, United Kingdom; for rumen NH_3 analysis) and one without (for VFA analysis).

Blood (9-mL/tube) was collected from all cows from the tail (coccygeal vein or artery) into 2 lithium heparinized blood tubes (Greiner Bio-One International GmbH, Kremsmünster, Austria). One blood tube was maintained as heparinized whole blood and the other was centrifuged (Megafuge 1.0R,

Heraeus Holding GmbH, Hanau, Germany), at 1,200 x g for 10 min at 4°C and plasma was removed and stored at -20°C until analysis. Urine was collected from all cows by valval stimulation and immediately acidified, and feces were obtained by rectal grab and stored at -20°C until analysis.

4.3.4 Intake Measurement

During the last week of pregnancy (19 July 2018 to 23 July 2018), individual animal intake of all cows was estimated using the dual marker method (Kartchner, 1981). This method is based upon an external marker being dosed to calculate faecal output (kg/d) and an internal marker used for determination of dry matter digestibility (DMD; %). The current experiment used titanium dioxide (TiO_2; Sigma-Aldrich New Zealand Co., Auckland, New Zealand) and indigestible acid detergent fibre (IADF) as external and internal markers, respectively. Titanium dioxide was bolused at a rate of 5 g (3 g Ti) per cow per d through gelatin capsules (Size 12; Torpac Inc., New Jersey, USA). After day 9, to allow for Ti to reach a steady state in the gastrointestinal tract, cows were faecal sampled twice daily, prior to receiving treatment (0700 h) and at 1600 h. Faecal samples were dried at 100°C, ground through a centrifugal mill to pass a 1-mm screen (ZM-200; Retsch, Haan, Germany), and composited by weight across day within animal. Additionally, three diet samples were collected from the baleage and a sample of the AHCS pellets were obtained, lyophilized, and ground to pass through a 1-mm screen. Baleage, AHCS pellets, and faecal samples were then used for IADF determination (described below) (Bohnert et al., 2002).

4.3.5 Sample Analysis

Rumen samples were analyzed for NH_3 by an enzymatic UV method (Neeley and Phillipson, 1988) using the Randox Rx Daytona (Cat. No. AM3979; Randox, Crumlin, United Kingdom). Rumen VFA concentrations were determined using a gas chromatograph (Chen and Lifschitz, 1989; Shimadzu GC-2010; Kyoto, Japan). Urea in plasma and urine were measured by a commercial enzymatic kinetic technique and analyzed using the Daytona RX Clinical Analyzer (Urea test kit Cat. No. UR9781; Randox, Crumlin, United Kingdom). Faecal samples were lyophilized and ground to pass through a 1-mm screen. Nitrogen content of faecal and urine samples were determined by combustion (Vario Max CN, Elementar

Analysensysteme, Hanau, Germany). Heparinized whole blood samples were analyzed for glutathione peroxidase (GPx) using an enzymatic method, according to the manufacture's specifications (RANSEL; Cat. No. RS504) and was analyzed using the Randox Rx Daytona. Plasma total antioxidant status (TAS; Cat. No. NX2332), non-esterified fatty acids (NEFA; Cat. No. FA115), and beta-hydroxybutyrate (BHBA; Cat. No. RB1008), were analyzed according to the Randox kit manual and analyzed using the Randox Rx Daytona.

Faecal samples obtained in the intake estimation period were analyzed for Ti by inductively coupled plasma optical emission spectrophotometer analysis (Varian 720 ICP-OES; Varian Australia Pty Ltd., Melbourne, Australia). Indigestible ADF in feces, baleage, and AHCS pellets was analyzed according to Bohnert et al. (2002) as implemented by (Beck et al., 2019b). Baleage and pelleted feed samples obtained during this period were analyzed for nutritive value using NIRS and are presented in Table 4.1. Prior to analysis calibration of NIRS were obtained for ash (method 942.05; AOAC, 1990), NDF (Van Soest et al., 1991) and ADF (method 973.18; AOAC, 1990), CP by combustion (Variomax CN Analyser Elementar), and DM digestibility (Iowerth et al., 1975). All calibration equations had $R^2 \geq 0.90$, and NIRS readings for the diet samples were within the calibration range.

4.3.6 Calculations

Faecal output, DMD, and intake were calculated using the equations of Kartchner (1981). Neutral detergent and acid detergent fibre digestibility (NDFD and ADFD) were calculated by the total NDF and ADF excreted in feces and their total intake.

4.3.7 Statistical Analysis

This experiment was designed as a completely randomized design with three treatments: SWO, SWP, or CON. As treatments and measurements were applied on the animal level, individual animal was the experimental unit. Samplings were performed periodically through the experiment and therefore the final statistical model included treatment, sampling date, and treatment by sampling date interaction as fixed effects. Additionally, day 0 values were used as covariates. For the data which was not repeatedly

measured (intake and digestibility) the statistical model only included fixed effect of treatment. Calving date was initially included in the model as a covariate, but was not significant (P > 0.10) for any of the models, and was subsequently removed. Least-square means were generated and, upon significance of the ANOVA, means separation was conducted by pairwise contrasts within sampling day (if appropriate) using the 'emmeans' package (Lenth, 2018) of R (R Core Team, 2020) (v.3.4.4). Statistical significance was declared at P ≤ 0.05 and tendencies were set at 0.05 < P ≤ 0.1.

4.4 Results

Treatments did not affect ($P \geq 0.58$) estimated intake and dry matter, NDF, or ADF digestibility (Table 4.2). Rumen fermentation characteristics results are presented in Table 4.3. In all instances, VFA concentrations, CON, SWO, and SWP were numerically lowest, intermediate, and highest, respectively. Cows on SWP had greater ($P < 0.05$) propionic and valeric acid concentration in ruminal fluids compared with CON, and SWO was intermediate and non-different ($P > 0.10$) from either treatment. Cows on SWP had 26% greater ($P < 0.05$) total VFA concentration than CON and again SWO was intermediate and non-different ($P > 0.10$) from the CON.

Table 4.4 presents the results for BCS, urine, and faecal. Due to stratification there was no difference in initial BCS of the cows ($P = 0.77$). Addition of both the extracts increased ($P < 0.01$) BCS compared with cows on CON. There were no differences detected in urinary N or urea, but SWP did have lower faecal N compared with CON and SWO ($P < 0.05$).

Figure 4.1 presents the blood chemical constituents. Differences in TAS were only apparent at the closest sampling dates to calving (i.e. the 80-d and 3-d post-calving sampling dates). Eighty days following initial treatment application, the SWO had greater ($P < 0.05$) TAS compared with both SWP and CON, and SWP was intermediate and greater ($P < 0.05$) than CON. After calving, the cows provided extracts had similar TAS ($P > 0.10$) and were greater than the CON ($P < 0.05$). There was no treatment by sampling day interaction ($P = 0.41$) for blood GPx activity, but the treatments were not different ($P > 0.10$) for the initial blood sample. Following the first application of treatments, there were significant

treatment effects for GPx activity. On d 38 and 80, CON had greater ($P < 0.05$) GPx activity compared with SWO and SWP. Three days after calving, CON still had the greatest GPx activity and was greater ($P < 0.05$) than SWP, but SWO was not different ($P > 0.10$) from CON or SWP. There was a sampling time by treatment interaction for NEFA ($P < 0.05$), reflecting no treatment differences except for 3-d after calving. After calving, CON had a greater ($P < 0.05$) NEFA concentration compared with those provided SWO and SWP. There was no treatment effect on BHBA concentration ($P = 0.46$), but it was affected by sampling day ($P < 0.01$), where BHBA concentration was increased following calving.

Across treatments and sampling days, NEFA and BHBA (r = 0.61; $P < 0.01$), GPx and NEFA (r = 0.48; $P < 0.01$), GPx and BHBA (r = 0.30; $P < 0.01$), and GPx and TAS (r = 0.22; $P = 0.04$) were all positively correlated. Furthermore, GPx and TAS were positively correlated (r = 0.38; $P < 0.01$) for the CON cows, but were not ($P > 0.10$) found to be correlated for the SWO and SWP treatment groups.

4.5 Discussion

We hypothesized that fermented plant extracts (SWO and SWP) would enhance rumen function by increasing ruminal fermentability, digestibility, DMI, and enhancing health related biomarkers. Based on the context of the present study and our results, we reject part of the hypothesis, as we were unable to detect treatment effects on diet digestibility or DMI. However, our results suggest that SWP had greater VFA concentrations for many of the individual VFA and had 26% greater total VFA concentration throughout the non-lactating period. Additionally, we did detect greater concentration in plasma antioxidant status (i.e. TAS) and reductions in oxidative (i.e. GPx) and metabolic stress (i.e. NEFA) during the transition period in cows provided SWO and SWP compared with the CON cows. Therefore, we accept the hypothesis that SWP altered ruminal fermentation and that SWO and SWP improved antioxidant status and reduced oxidative and metabolic stress, which would imply enhanced animal welfare.

The increased rumen fermentation of SWP cows, as evidenced by 26% greater total VFA concentration compared with CON cows, appears to be independent of DMI or DMD, as these feed parameters were not different between cow treatment groups. Greater ruminal VFA concentration does not necessarily mean greater energy supply from a given unit of intake nor even greater total VFA production per unit of intake, due to potential differences in VFA absorption across the rumen wall (Dijkstra et al., 1993) or due to differences in ruminal liquid volume (Hall et al., 2015). However, cows receiving SWO or SWP had greater BCS change compared with CON cows, which supports the greater VFA concentrations and suggests that SWP increased energy supply by enhancing rumen function with a more efficient fermentation.

Nitrogen metabolism was not affected by treatments during the gestation period, as evidenced by the lack of differences in ruminal NH_3 or urinary N concentrations. Such a lack of treatment effect indicates that there were no differences in fermentation of protein in the rumen. Previous work by Beck et al. (2019), however, reported that SWO products affect *in vitro* fermentation by reducing ruminal NH_3 production by 6%. This discrepancy between these two experiments may relate to the diluted concentration of ruminal fluid used in the *in vitro* technique, which places ruminal fluid and buffer at a 1:5 ratios. This would mean that a greater amount of SWO was applied relative to ruminal fluid during the *in vitro* experiment compared with the current *in vivo* experiment, indicating that the fermented plant extracts at a greater dose may alter ruminal fermentation to reduce urinary N excretion.

We analyzed the effect of SWO and SWP on TAS and GPx activity, which increased around calving. We can conclude that the 5-mL/d dose used during gestation improved antioxidant status and reduced oxidative stress based on the results of the d 80 sampling. However, we cannot say whether the improved antioxidant status and reduced oxidative stress measured 3 d after calving was a result of the increased (100-mL/d) dose or a carry-on from the gestation period. Total antioxidant status functions as a biological marker to measure the “all encompassing” antioxidant capacity in biological samples, and thus may provide a better estimate than simply measuring individual antioxidants (Ghiselli et al., 2000).

Additionally, GPx is an important antioxidant enzyme and as such has been suggested to provide an estimate of oxidative stress (Celi, 2011). Blood GPx is responsible for the defense against oxidative damage for animal tissues and has been identified as a marker of oxidative stress (Celi, 2011). The blood GPx activity was much greater in the CON cows than in their SWO and SWP counterparts. Our interpretation of this result is that SWP and SWO cows were under less oxidative stress than the CON cows, which likewise fits with the observed TAS results and supports the concept of (Beck and Gregorini, 2020) and our present hypothesis. This interpretation was also proposed by Guo et al. (2013), who reported increased levels of GPx when subacute ruminal acidosis was induced in dairy cows. Additionally, Bernabucci et al. (2005) concluded that transition dairy cows with the greatest BCS loss had greater GPx activity, thereby indicating oxidative stress.

Oxidative stress has been shown to relate to the majority of the metabolic disorders associated with transitioning high producing dairy cows. In an extensive review on the relationship between oxidative stress and disease in livestock, Lykkesfeldt and Svendsen (2007) discussed the link between oxidative stress and mastitis and pneumonia in ruminants. Additionally, others have suggested links between oxidative stress and metritis, mammary edema, mastitis, and retained fetal membranes (Sordillo and Aitken, 2009). As such, biomarkers of oxidative stress and antioxidant status have been identified and reported to be promising predictors of future illness and therefore welfare of cattle (Beck and Gregorini, 2020). Wisnieski et al. (2019) explored the predictive potential of oxidative stress, inflammatory, and nutrient metabolism biomarkers for disorders and diseases common to transition dairy cows. It was determined that models associated with oxidative stress and inflammatory markers measured at dry off had greater predictive ability than nutrient metabolites. However, a combined model containing oxidative stress, inflammatory, and nutrient metabolism markers had the highest predictive ability compared to any model that utilized each of these classes of markers individually (Wisnieski et al., 2019).

Calving time is a stressful event for animals, but especially for high producing dairy cows (Sordillo and Mavangira, 2014). Not only do dairy cows have the physiological stress associated with parturition, which all animals experience, but also experience large increases in energy demand as a result of onset of lactation (Goff and Horst, 1997). As such, both NEFA (measure of fatty acid mobilization) and BHBA (measure of ketone body accumulation) concentrations increased for all treatments after calving (Figure 4.1), thereby providing direct evidence that these animals were experiencing metabolic stress. As with the current experiment, antioxidant supplements often do not show benefits for certain biomarkers until after a stressful event. For example, when sheep were provided antioxidants (vitamin E and selenium) at supra-nutritional doses, differences between control sheep and treated sheep were only apparent following an imposed challenge of heat stress (Chauhan et al., 2014). This is apparent in the current experiment especially for the TAS biomarker, with treatment differences only detected at the sampling dates closest to calving.

Non-esterified fatty acids are a measure of fat mobilization, as they are fatty acids that are not esterified to glycerol backbones. Following conversion to acetyl-CoA units, through beta-oxidation, carbon derived from NEFA can enter into the citric acid (TCA) cycle; however, this is dependent on oxaloacetate, whose availability is based on glucogenic precursors (Van Soest, 1994). In situations where metabolic demands are at a rate where fat mobilization outpaces the ability of acetyl-CoA to be incorporated into the TCA cycle, ketone bodies, such as BHBA, concentrations increase (Van Soest, 1994). This can be seen in our current experiment where BHBA and NEFA concentrations increase around calving (Figure 4.1). In fact, NEFA and BHBA concentrations were correlated ($r = 0.61$) in the current study. When ketone bodies reach a great enough concentration, ketosis can occur. For example, Li et al. (Li et al., 2016) determined that ketotic and healthy cows had serum BHBA concentrations of 3.09 ± 0.13 and 0.49 ± 0.11, respectively. Subsequently, BHBA and NEFA have been suggested as biological markers to predict metabolic disorders in transition dairy cows (Wisnieski et al., 2019). Even with the differences observed in our treatments, none of the cows experienced metabolic disorders.

Even though none of the cows experienced metabolic disorders, the reduced NEFA concentrations in the SWO and SWP treatments compared with the CON is promising and may indicate that SWO and SWP can reduce the incidence of metabolic disorders in lactating dairy cows.

The metabolites related to mobilization of fat stores (i.e. NEFA and BHBA) have been associated with oxidative status in ruminants (Sordillo and Aitken, 2009; Sordillo and Mavangira, 2014). Non-esterified fatty acids are especially related to oxidative stress and can produce oxidants through several means. The first is during the beta-oxidation step, which breaks down fatty acids to acetyl-CoA units (Quijano et al., 2016). Another means by which NEFA production can induce oxidative stress is through its effects on albumin, which is the major plasma carrier of NEFA and is itself an antioxidant. As NEFA binds to albumin, copper-albumin complexes can be converted from antioxidants to pro-oxidants (Gryzunov et al., 2003). This relationship is evident in the current experiment. Glutathione peroxidase activity across all sampling days was positively correlated to NEFA ($r = 0.48$) and, to a lesser extent, to BHBA ($r = 0.30$). Even with the positive relationships between GPx activity with NEFA and BHBA, indicating more NEFA and BHBA resulting in greater oxidative stress, GPx and TAS were also positively correlated across treatments and sampling days ($r = 0.22$). However, upon further investigation, this positive correlation was only significant for the CON treatment ($r = 0.38$), indicating that as greater fat mobilization occurred (CON had greater NEFA levels), there was an associated larger demand for GPx activity to account for the increased oxidants. Therefore, within the CON treatment only, as GPx activity increased a subsequent increase in TAS occurred by way of GPx providing antioxidant support. Consequently, it is proposed that increased GPx activity was a result of increased oxidative stress; however, increased GPx activity in oxidative stressed animals (i.e. the CON animals) was associated with an increased TAS. Finally, greater oxidative stress can induce further lipolysis, which leads to even greater NEFA production, thereby creating a vicious cycle (Abuelo et al., 2015), explaining why the CON cows had a greater concentration of NEFA compared with the other treatments, without subsequent differences in BHBA concentrations between treatments (Figure 4.1).

There exist several potential modes of action for the SWO and SWP fermented plant extracts to increase antioxidant status. The first is by the phytochemicals extracted from the seaweed (SWO and SWP) and from the terrestrial plants (SWP only) as phytochemicals, especially phenolic compounds, which are potent antioxidants (Gessner et al., 2017). Several modes of action for phytochemicals to provide *in vivo* antioxidant support have been suggested. Beck and Gregorini (2020) suggested that unabsorbed phytochemicals may improve antioxidant status by reducing oxidants in the gastrointestinal tract and that absorbed phytochemicals may act directly at the tissue level to provide either direct intervention against oxidants or to regulate gene expression to increase antioxidant defense. Another mode of action may be through the fermentation products of microorganisms, as the products used in the current experiment are fermented extracts. Ran et al. (2019) provided a lactobacillus fermentation products to finishing steers in a feedlot at the same dose (5 mL/hd/d) provided to the dry cows in this study. Additionally, a probiotic supplement composed of *Lactobacillus plantarium* improved serum and ruminal antioxidant activity in lambs after weaning (Izuddin et al., 2020). Our current results agree with Ran et al. 2019 and Izuddin et al. 2020 in that the lactobacillus fermentation product enhanced biomarkers in a manner indicating improved antioxidant status in the growing steers and lambs, respectively.

The final mode of action may be related to the increased fermentation and BCS and reduced NEFA (around calving) by the cows provided the extracts. As discussed above, it is well established that plane of nutrition and level of lipid mobilization has a strong relationship with oxidative stress (Sordillo and Aitken, 2009; Sordillo and Mavangira, 2014). For example, Bernabucci et al. (Bernabucci et al., 2005) determined that cows with greater BCS loss resulted in greater NEFA concentration and subsequent oxidative stress. Additionally, goats had lower antioxidant capacity at parturition when they were fed a lower energy density diet (5.6% corn) compared with a higher energy diet (19.7% corn) (Celi et al., 2010). We did not detect differences in intake or digestibility, which would be expected with greater ruminal fermentation products. Additionally, differences in NEFA were only detected after calving, so that

differences in oxidative status seen prior to calving (i.e. sampling day 38) may not be explained by plane of nutrition, but may be from one of the previously outlined modes-of-action.

4.6 Conclusions

This experiment is the first to show a fermented seaweed alone (SWO) and a seaweed plus terrestrial plant (SWP) extracts affect ruminal fermentation and blood constituents which are related to metabolic disorders associated with transition dairy cows. Based on our results we conclude that SWP can increase ruminal VFA concentration across the dry period resulting in greater rumen fermentation and thereby animal performance, while reducing oxidative stress and improving antioxidant status compared to the CON treatment. Likewise, the SWP and SWO treatments reduced NEFA concentration around calving. These results taken collectively highlight the ability for these plant extracts to improve ruminal fermentation, increase BCS, and improve health of dairy cows across the last third of gestation and the transition from non-lactating to lactating periods.

Table 4.1 Diet chemical characteristics determined by near infrared spectroscopy

Item, % DM [a]	Lucerne Silage	Ryegrass	AHCS Bait [b]
DM, % as-fed	48.55	17.00	
NDF	41.38	39.27	39.06
ADF	33.20	20.36	21.28
CP	20.69	18.61	15.69
DMD	63.35	79.30	75.88

[a] DM = Dry Matter, NDF = Neutral detergent fiber, ADF= acid detergent fiber, CP = Crude protein, DMD = dry matter digestibility.

[b] AHCS Bait = pelleted supplement provided from the automated head chamber systems used to estimate gaseous emissions.

Table 4.2 Diet intake, energy intake, and fiber fraction digestibility of dry cows either not supplemented (CON) or supplemented with fermented plant extracts (SWO and SWP). Intake was measured from 19 July through 23 July 2018. Using the dual marker technique (indigestible ADF, as internal marker; titanium, as external marker).

	Treatments				*P*-value
Item [a]	CON	SWO	SWP	SEM[b]	Treatment
n	9	9	9	—	—
Silage intake, kg/d	14.03	14.43	14.76	0.82	0.82
AHCS bait intake, kg/d	0.65	0.39	0.44	0.09	0.12
Total intake, kg/d	14.68	14.83	15.20	0.80	0.90
DMD, %	64.30	64.46	64.82	0.54	0.79
OMD, %	68.95	68.78	69.51	0.68	0.73
NDF digestibility, %	59.70	60.34	61.05	0.91	0.58
ADF digestibility, %	60.75	61.37	61.47	0.87	0.82

[a] AHCS Bait = pelleted supplement provided from the automated head chamber systems used to estimate gaseous emissions; DMD= Dry matter digestibility determined by an internal marker (indigestible ADF); OMD = organic matter digestibility; NDF = Neutral detergent fiber; ADF = Acid detergent fiber.

[b] SEM = Standard error of the mean

Table 4.3 Rumen fermentation characteristics (estimated marginal means) as influenced by two types of fermented plant extracts (SWO and SWP).

	Treatments				*P*-value[e]		
Item, mmol/L[d]	CON	SWO	SWP	SEM[f]	TRT	Day	TRT×Day
n	3	3	3	---	---	---	---
NH_3	6.65	6.87	6.52	0.43	0.95	0.07	0.37
Acetic	36.60	39.47	47.58	3.11	0.06	<0.01	0.38
Propionic	7.74[b]	8.36[b]	10.36[a]	0.79	0.04	0.05	0.60
Butyric	3.71	3.95	4.98	0.44	0.07	<0.01	0.68
Valeric	0.46[b]	0.53[ab]	0.63[a]	0.04	0.02	<0.01	0.39
Hexanoic	0.07	0.14	0.17	0.04	0.19	<0.01	0.74
Iso-Butyric	0.89	0.91	0.93	0.04	0.28	0.06	0.43
Iso-Valeric	1.11	1.14	1.17	0.08	0.21	0.05	0.78
Ace.:Prop.	4.86	4.80	4.49	0.13	0.13	0.05	0.60
Total VFA	50.99[b]	55.48[ab]	64.43[a]	4.65	0.05	<0.01	0.45

[a-c] means within a row with different superscripts differ ($P \leq 0.05$).

[d] NH_3 = ruminal ammonia; Ace.:Prop. = acetate-to-propionate ratio; Total VFA = sum of all volatile fatty acids concentration

[e] TRT = treatment; TRTxDay = the treatment by day interaction

[f] SEM = Standard error of the mean

Table 4.4 Animal responses to either no addition (CON) or the addition of fermented plant extracts (SWO and SWP). Samples were repeatedly measured at 0, 38, and 80 days following first application of the treatments (7 May 2018).

	Treatments				*P*-value[e]		
Item[d]	CON	SWO	SWP	SEM[f]	TRT	Day	TRT×Day
n	8	8	8	---	---	---	---
Initial BCS	4.25	4.31	4.19	0.12	0.77	---	---
BCS	4.27[b]	4.43[a]	4.47[a]	0.04	<0.01	0.02	0.95
Urine Urea	109.66	139.47	109.87	17.4	0.24	<0.01	0.87
Urine N, %	3.6	4.9	3.8	0.6	0.24	<0.01	0.96
Fecal N, % DM	2.10[a]	2.06[a]	1.89[b]	0.06	<0.01	<0.01	0.51

[a-c] means within a row with different superscripts differ ($P \leq 0.05$).

[d] BCS = body condition score; Urine Urea (mmol/L); N = nitrogen

[e] TRT = treatment; TRTxDay = the treatment by day interaction

[f] SEM = Standard error of the mean

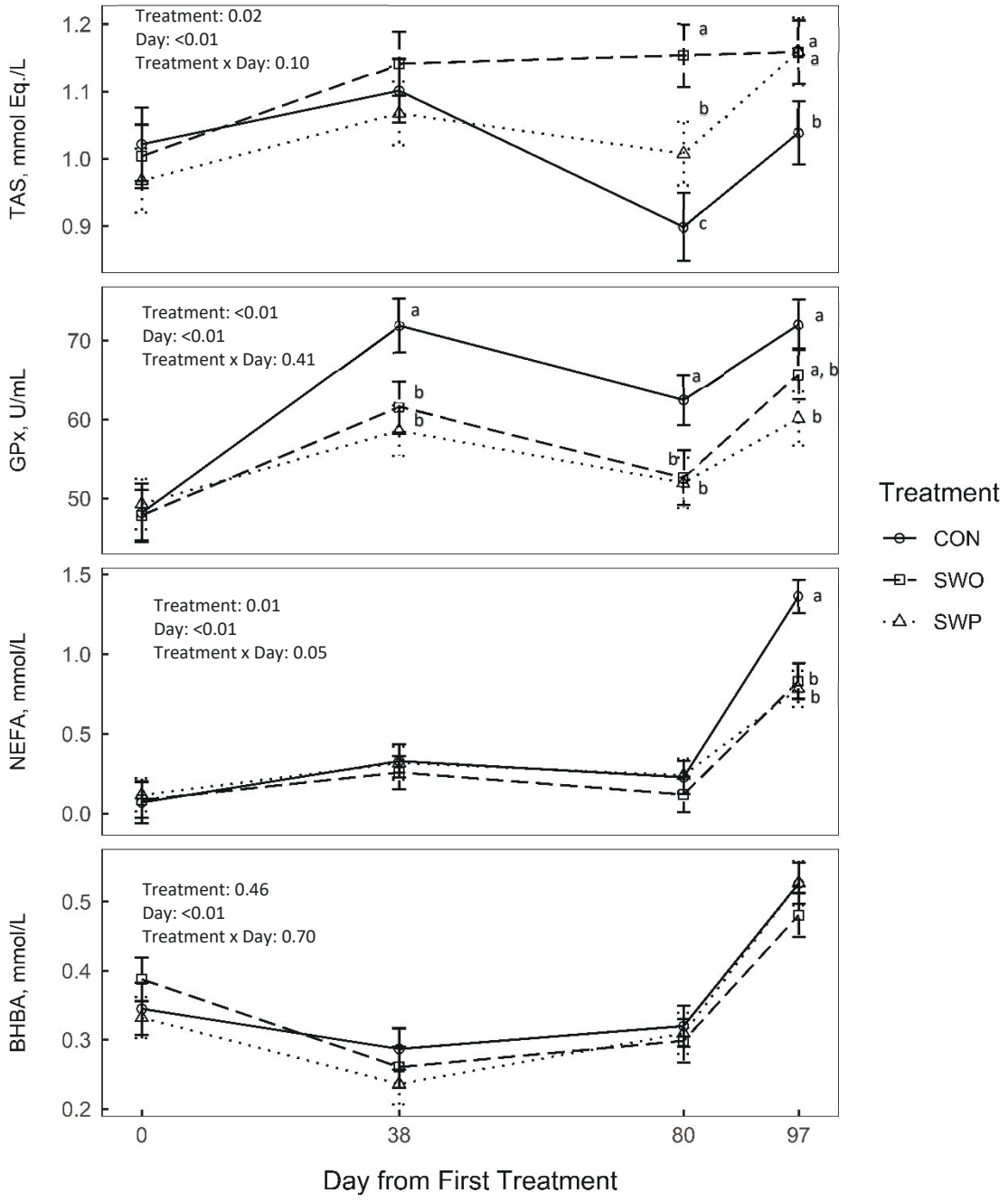

Figure 4.1 Blood characteristics of transition dairy cows provided either a daily oral drench of water (CON), a seaweed extract (SWO), and a seaweed plus terrestrial plants extract (SWP). Metrics measured included total antioxidant status (TAS), glutathione peroxidase activity (GPx), non-esterified fatty acids (NEFA), and beta-hydroxybutyrate (BHBA). Blood samples were collected prior to treatment application (day 0), 38 and 80 d of receiving treatment, and 3-d after calving (97 ± 6 d after receiving treatment).

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Chapter 5

Fermented plant extracts could reduce nitrogen excretion from grazing lactating dairy cows.

5.1 Abstract

The objective of this experiment was to determine the effect of a fermented seaweed (*Ecklonia radiata*) extract product (SWE; Animal Health Tonic; AgriSea; Paeroa, New Zealand) and a fermented seaweed plus terrestrial plant extract (SWP; Aleviate; AgriSea, Paeroa, New Zealand) on milk production, dry matter intake (DMI), diet digestibility, and urinary N excretions and enteric methane (CH_4) emissions. Holstein-Friesian × Jersey cross pregnant dairy cows (n = 23; body weight = 488.8 ± 47.6, mean ± SD) were allocated to receive daily oral doses of water (CON), SWE, or SWP. During the non-lactating period (i.e. last third of gestation) the cows received 5-mL of their treatments once daily and this dose was increased to 100-mL per cow per d following calving. Blood samples were taken prior to applying treatments (d 0), d 39, d 80, and 3-days after calving. Following calving, cows were maintained in a colostrum herd for 5-d and then monitored for two weeks within a milking herd to measure their lactation performance. There were no differences ($P > 0.10$) between treatments on DMI or DM, NDF, or ADF digestibility, but there was a tendency for SWP to have a greater ($P < 0.10$) crude protein (CP) digestibility compared with SWE. There was no ($P > 0.10$) treatment effects detected on milk production values, except ($P < 0.01$) for milk lactose concentration where SWP had a lower concentration than SWE and CON. There was no treatment ($P > 0.10$) effects on CH_4 or carbon dioxide (CO_2) emissions. There were no treatment differences for plasma urea nitrogen (PUN) concentration throughout the dry period, but 3-d after calving CON had the greatest ($P < 0.05$) PUN concentration, and the PUN concentration for SWP was intermediate and greater ($P < 0.05$) than SWE. This may indicate a dose dependent effect of the plant extracts on N dynamics as the dose was increased following calving. Further, the SWP treatment

had the lowest concentration of milk urea N and was significantly ($P < 0.05$) less than CON. The cows provided SWE had numerically intermediate concentrations of milk urea N and tended ($P = 0.07$) to be lower than CON, and was not different from SWP ($P = 0.52$). Accordingly, when daily urinary N excretion was calculated, SWP had 18.1% lower ($P < 0.05$) urinary N excretion than CON and SWE had a tendency ($P = 0.07$) to have 14.0% lower urinary N excretion than CON, while SWE and SWP did not differ ($P > 0.10$). These results indicate that by using seaweed containing fermented plant extracts, the environmental impacts associated with intensive grazing dairy systems can be reduced without reducing forage intake or sacrificing milk production.

Keywords: Seaweed extract, plant extract, Nitrogen excretions

5.2 Introduction

Unlike most other developed countries, the largest contributor to greenhouse gases (GHG) in New Zealand is from agriculture, which accounts for 48.1% of total emissions (Ministry for the Environment, 2019). The emissions from agriculture are largely from soils (namely from nitrous oxide-emissions, with 63.5% of soil N_2O emissions arising from livestock urine deposited onto soil), and enteric methane (CH_4). Agricultural soils and enteric CH_4 represent 10.6 and 34.2% of New Zealand's total GHG emissions, respectively. Much of the emissions from New Zealand agriculture arise form intensive grazing dairy systems. These systems often provide cows with diets that have nitrogen (**N**) levels far above their requirements, which leads to greater urinary N (**UN**) excretion. For example, when Holstein heifers were fed increasing levels of dietary N (1.45, 1.89, 2.50, 2.97, and 3.40% of DM) there were linear increases in daily UN excretion (21.7, 36.1, 68.7, 94.3, and 120.8 g UN per d, respectively) with the majority (92-99%) of the additional UN in the form of urea (Marini and Van Amburgh, 2003). Further, a perennial ryegrass (*Lolium perenne*) based sward, which are typical in intensive grazing dairy systems located in temperate regions (e.g. New Zealand), may provide N content exceeding 4% of DM (Di and Cameron, 2007). This leads to low N use efficiency by the animal and N loading rates at the urine patch

level can be as high as 1,000 kg N per ha. Subsequently, this rate of N deposition surpasses the ability of sward plants to utilize it and it is lost from the system either through leaching or by emissions from the soil, which are environmental pollutants (Cameron et al., 2013). Additionally, enteric CH_4 emissions from grazing ruminants are associated as a significant source of anthropogenic greenhouse gases. Enteric CH_4 represents 39.1% of global livestock GHG emissions (Gerber et al., 2013), which is partly due to its greater ability to trap heat compared with CO_2 (28-times greater global warming potential; IPCC, 2013). These environmental impacts associated with ruminant livestock production need to be addressed.

Plant secondary compounds and plant extracts represent one potential opportunity to reduce the environmental concerns associated with ruminant production. For example, tannins (from terrestrial and aquatic plants) have a strong ability to precipitate N and shift N from urine to feces (Waghorn, 2008). Tannins may also reduce enteric CH_4 emissions by reducing availability of substrate for microbes in the rumen (Carulla et al., 2005; Grainger et al., 2009) or by directly reducing the abundance or metabolic activity of methanogenic bacteria (Tan et al., 2011). Furthermore, seaweeds and seaweed extracts have been shown to reduce ruminal NH_3 production *in vitro* (Wang et al., 2008; Beck et al., 2019), which may lead to reduced urinary N excretion, and enteric CH_4 emissions (Machado et al., 2014; Li et al., 2016; Maia et al., 2016; Roque et al., 2019). Thus, both terrestrial and aquatic plant secondary compounds and plant extracts provide an opportunity for reducing negative environmental impacts from grazing-based ruminant production.

Previously published *in vitro* experiments on a fermented seaweed (*Ecklonia radiata*) extract (**SWE**; Animal Health Tonic; Agrisea Ltd., Paeroa, New Zealand) were conducted to determine an appropriate dose rate: mL of extract per kg of dry matter intake (DMI; Beck et al., 2019). This preliminary study determined that the smallest addition of SWE (5 mL per cow per d) resulted in a 5-6% reduction in ruminal-NH_3 production. Ruminal fermentation of proteins produces NH_3 as a by-product, which is rapidly transported across the rumen wall to the liver where it is converted to urea, through ureagenesis, and subsequently recycled to the rumen or excreted in the urine. Therefore, Beck et al. (2019) concluded

that further research would be required to determine if the reduction in NH_3 production would relate to biologically significant lower urine N excretions. There has yet to be any research conducted on SWE or SWP to determine their effects on milk production. Therefore, the objective of this experiment was to determine if SWE or SWP would influence DMI, diet digestibility, milk production, UN excretion, and CH_4 emissions from dairy cows in non- and early lactation. We hypothesized that these plant extracts would increase milk production and reduce UN excretion and CH_4 emissions, without altering DMI.

5.3 Materials and Methods

The procedures outlined in this manuscript were approved by the Lincoln University Animal Ethic Committee (AEC 2018-08). The experiment described is a continuation of an experiment, with some results reported elsewhere (Chapter 4).

5.3.1 Animals and Handling

This experiment was conducted from 7 May to 9 September 2018 at the Lincoln University Research Dairy Farm. This experiment was conducted on Holstein-Friesian × Jersey cross dairy cows over the non-lactating period and into the first two weeks of lactation. The management of these animals from the start of treatment application to three days post-calving has been described previously (Chapter 4). In brief, dairy cows [n = 27 (18 non-cannulated and 9 cannulated); 4.43 ± 8.16 body condition score (BCS); 10 August 2018 ± 8 d expected calving date; Mean ± SD] were stratified by BCS (1 to 10 scale; DairyNZ, 2018) and then randomly allocated to one of three treatments. The treatments imposed on the dairy cows were a daily oral dose of either water (CON), a commercial fermented seaweed (*Ecklonia radiata*) extract (SWE; trade name: Animal Health Tonic, Agrisea Ltd, Paeroa, New Zealand), or a commercial fermented seaweed and terrestrial plants extract (SWP; trade name: Aleviate; Agrisea Ltd., Paeroa, New Zealand). Over the non-lactating period, cows were dosed 5 mL per cow per d with their respective treatment and this was diluted (1:3) with water so that each animal received a 20 mL oral drench. Following calving, cows were provided 100 mL per cow per d until the end of the experiment. The dose was increased from 5 to 100 mL because cows can experience a 20-fold increase in

energy requirements during the onset of lactation (Grummer, 1995). From 7 May 2018 to 26 July 2018, cows were maintained in a grass-free outdoor enclosure and fed lucerne bailage at 17 kg DM/cow per d, with the assumption that 20% of the bailage would be wasted, and 14 kg DM/cow per d would be consumed.

Approximately two weeks before calving (26 July, 2018), cows were moved to a perennial ryegrass (*Lolium perenne* L.) dominant paddock. During the pre-calving phase, cows were maintained in a dry herd until after calving, after which they were maintained in a colostrum herd for five days and subsequently moved to a milking herd, from when milk production data was collected for 14 days. Dry cows were provided 16 kg ryegrass pasture DM/cow per d above 3-cm. Following calving, cows were provided 30 kg ryegrass pasture DM/cow per d above ground level. Herbage allowance was ascertained by determining forage mass (kg/ha), using a rising plate meter (Jenquip; Feilding, New Zealand) and the manufacturers' pre-established equations. The required area was then allocated to the cows using temporary electric fencing. Herbage samples were taken 3-times throughout the experiment by hand-clipping herbage within a 0.10 m^2 area in 3 random locations. The bailage (fed in the non-lactating period) and forage samples were frozen at -20°C, freeze-dried, and ground to pass through a 1 mm screen using a centrifugal mill (ZM200 Retsch). After calving, cows were milked twice daily at 0600 and 1500 h. Individual milk yield was measured during each milking using an automated milking system (DeLaval Alpro Herd Management System, DeLaval, Tumba, Sweden).

5.3.2 Marker Dosing for Intake Measurements

The dual marker technique was used to estimate forage DMI during the dry and lactation periods of the experiment. This method utilizes an internal maker (i.e. indigestible component found in feed) and an external marker (i.e. indigestible marker provided at a known dose; Garrett et al., 2020). Within the current experiment, indigestible acid detergent fiber (**IADF**) was used as the internal marker and titanium dioxide (**TiO_2**) was used as the external marker. The DMI, and digestibility of cows in the non-lactating period have been previously described and are reported in Chapter 4. Once cows moved from

the colostrum herd to the milking herd (5 d post-calving), they received a daily dose of 5-g of Titanium dioxide (**TiO_2**) via a bolus (Size 12; Torpac Inc., New Jersey, USA) after the morning milking, until they were removed from the experiment (19 d post-calving). After 9 d of receiving the TiO_2, fecal samples were taken twice daily after each milking for 5 d, until the cows were removed from the experiment. Fecal samples were oven-dried (60°C) and ground to pass through a 1-mm screen by a centrifugal mill (ZM200 Retsch).

5.3.3 Methane Measurements

The automated head chamber system (AHCS) on a trailer with batteries and generator that can be used in rotational grazing systems was used to determine daily enteric gaseous emissions (Jonker et al., 2017). It is generally suggested to begin training animals to the units as much as 4-wk prior to the beginning of an experiment using around 20% more animals during the acclimation period (Gunter and Beck, 2018). However, the cows used for this experiment were unavailable until 3-d prior to the start and the standard gases used for calibrations were unavailable until d 30 (6 June, 2018). Due to these reasons, training the cows to use the AHCS occurred during the beginning (d 0 to d 30) of the experiment and only data obtained after calibrations on d 30 was used for analysis of gas flux over the non-lactating period. Following calving and when cows were moved to the lactation herd they were managed alongside an AHCS, of which they had access to for the remainder of the experiment.

Once operational, sensor calibrations were performed using the AHCS's built in auto-calibration system at 0420 h each day. The unit's gas recovery was determined by releasing CO_2 from a CO_2 cylinder for 3 min. Gravimetric release of CO_2 was compared with the CO_2 production determined by the AHCS and recovery was 102.7% during the dry (SD = 7.1; n = 5) and 98.9% during lactation (SD = 11.3; n = 5) periods. The CO_2 recoveries were performed at the start of the measurement period during the non-lactating and lactating portion of the experiment. Finally, the AHCS air-filter was changed when the airflow rate dropped below what is recommended (26 L/s; Gunter et al., 2017).

The AHCS unit functions by dropping a pelleted supplement (31 g/drop, SD = 1.25, n = 15) and measuring gas emissions while the animal is consuming the supplement. The pelleted supplement used in the current experiment was a commercial product (7 mm diameter; Alpaca and Llama pellets; Dunstan Nutrition, Hamilton, New Zealand). For this experiment the unit was set to drop feed at 25 sec intervals, dispense 8 drops per visit, have a 3 h minimum allowable time between visits for each cow, and to have a maximum of 8 visits per d. These settings were applied to keep the animal at the AHCS for 3 min or longer as it has been recommended that only visits with 3 min durations should be used for analysis (Arthur et al., 2017; Gunter and Beck, 2018). All animals used in the non-lactating and lactating phases had greater than the 20 recommended visits to the AHCS for accurate emission estimates (98.6 ± 13.4 and 22.8 ± 1.8; mean ± standard error of the mean, respectively) by some (CITATION). The spot samples measured during this experiment were averaged for each animal, within the dry (30 d of measurements) and lactation periods (14 d during the measurement period), to provide one measured value per animal per period and only non-cannulated animals were used for analysis.

5.3.4 Animal Sample Collection

Over the non-lactating period, blood samples were collected on d 0 (7 May 2018), 38 (14 June 2018), and 80 (26 July 2018) of treatment application, in the morning (0700) prior to feeding. Three days after calving (13 August 2018 ± 8 d) a blood sample was collected after the morning milking. Blood (~10 mL) was drawn into evacuated heparinized blood tubes and was centrifuged at 1,200 × *g* for 10 minutes at 4°C. Plasma was then aspirated, transferred into 2 mL Eppendorf tubes and stored at -20°C until further analysis. On day 7 and 14 of each respective cow's lactation phase, milk samples were collected from both morning and afternoon milking. A subsample of this milk was centrifuged at room temperature (20°C) for 10 min at 4,000 × *g* to separate the milk fat, and 1 mL of the skimmed milk was aspirated, transferred into a 2 mL Eppendorf tube, and stored at -20°C until further analysis.

5.3.5 Sample Analysis

A subsample of fresh herbage was dried at 60°C for the determination of DM content. The freeze-dried bailage and forage were analyzed by near-infrared spectroscopy (**NIRS**) for nutritive quality. Prior to analysis with the NIRS, calibration equations were developed for ash (method 942.05; AOAC, 1990), neutral detergent fiber (NDF; Van Soest et al., 1991) and acid detergent fiber (ADF; method 973.18; AOAC, 1990), CP (Variomax CN Analyser Elementar), and DM digestibility (Iowerth et al., 1975). All equations had R^2 values ≥ 0.9. The fecal samples were composited by weight across sampling time and date for each animal. Composited fecal samples, forage, and AHCS pellets were analyzed for IADF (Bohnert et al., 2002; Beck et al., 2019). Fecal samples were analyzed for Ti concentration by inductively coupled plasma optical emission spectrophotometer (Varian 720 ICP-OES; Varian Australia Pty Ltd., Melbourne, Australia). Fecal samples were further analyzed for ash (method 942.05; AOAC, 1990), CP by combustion (Variomax CN Analyser Elementar), NDF (Van Soest et al., 1991) and ADF (method 973.18; AOAC, 1990). Milk and plasma urea concentrations were analyzed using an enzymatic kinetic technique by an automatic clinical analyzer (Urea test kit; Randox Daytona; Randox, Nishinomiya, Japan). Finally, milk was analyzed for fat, protein, and lactose content using MilkoScan (FOSS NIRS Systems 5000; Maryland, USA).

5.3.6 Calculations

Fecal output and DM digestibility (DMD) were calculated using the external and internal markers, respectively (Kartchner, 1981). The calculations provided by Kartchner (1981) describe how to account for supplement intake to determine forage DMI. Based on the total estimated NDF, ADF, and CP intake and their total excretion, the digestibility of these nutritive components was calculated. Daily UN excretion was calculated for both weeks of lactation based on the average of the morning and afternoon milk urea N (**MUN**) concentration (Kauffman and St-Pierre, 2001; Kohn et al., 2002). The calculation is:

$$UN, \frac{g}{d} = 0.0259 \times BW(kg) \times MUN(\mathrm{mg/dL}) \quad [1]$$

5.3.7 Statistical Analysis

This experiment utilized a completely randomized design. To analyze body condition score (**BCS)** and average body weight, treatment only was used as a fixed effect. Dry matter intake, apparent DMD, NDF digestibility, ADF digestibility, CP digestibility, and gas emissions were analyzed using treatment, stage (i.e. dry or lactating), and their interaction as fixed effects. Plasma urea concentration was analyzed using a fixed effects model with repeated measures, with treatment, sampling date, and the treatment-by-sampling date interaction as fixed effects. Likewise, milk production, milk characteristics, milk urea, and calculated UN excretion was collected for week one and two of lactation, and therefore the statistical model for these variables included treatment, lactation week, and their interaction as fixed effects. An analysis of variance (**AOV**) was generated using the 'aov' function of base R (R Core Team, 2018, v.3.4.4). Additionally, least-squares means were generated and, following significance of the AOV, means separation was done using the 'emmeans' function (Lenth, 2018). Finally, Pearson's correlations between DMI and CH_4 emissions, CH_4 yield, milk yield, and protein yield and plasma urea N measured 3 d after calving and MUN were generated using the 'cor.test' function of the base R software. All statistical analysis was conducted using the R software (R Core Team, 2018, v.3.4.4), statistical differences were considered significant if $P \leq 0.05$, and tendencies were declared when $0.05 < P \leq 0.1$.

5.4 Results

Nutritive quality predicted by NIRS of bailage, herbage, and the AHCS pellets are presented in Table 5.1. There were no differences detected between the SWE, SWP, and CON treatments for BCS or initial body weight (Table 5.2). There were no treatment main effects ($P \geq 0.20$) or treatment by measurement period (non-lactating and lactating) interactions ($P \geq 0.43$) detected for DMI or DM, OM, NDF, ADF, and CP digestibility (Table 5.3). By design, non-lactating cows consumed less lucerne bailage and less total DM compared with ryegrass herbage fed lactating cows ($P < 0.01$). The amount of supplement from the AHCS did not differ ($P = 0.67$) between the measurement phases. The lactating cows fed ryegrass dominant swards had greater DM, OM, NDF, ADF and CP digestibility ($P < 0.01$)

compared with when the cows were non-lactating and fed lucerne bailage. Milk yield and composition was not different ($P \geq 0.44$) for all variables, with the exception of lactose and MUN (Table 5.4). The SWP treatment group had less lactose concentration in their milk compared with the CON and SWE treatment groups, while the CON and SWE were not different ($P = 0.32$). Cows provided the SWP had 17.0% less ($P = 0.02$) MUN concentration than the CON, while SWE had a tendency ($P = 0.07$) to have 12.9% less MUN than the CON.

There were no ($P > 0.10$) treatment differences observed for any of the gas emissions estimates during the non-lactating or lactating phases (Table 5.5). There were no ($P \geq 0.35$) treatment differences for N intake, fecal N excretion (g/d), milk N excretion (g/d), or apparent N retention; however, there were numeric differences for apparent N retention (Table 5.6). When using MUN to calculate daily UN excretion (g UN/d), SWP excreted 18.1% less ($P < 0.05$) than CON. Further, SWE tended ($P = 0.07$) to excrete 14.0% less UN per d than the CON. Over the dry period, there were no ($P > 0.10$) treatment effects on plasma urea concentration; however, after calving, CON was greater ($P < 0.05$) than the cows provided the extracts and SWP was intermediate and greater ($P < 0.05$) than SWE (Figure 5.1).

5.5 Discussion

The hypothesis was that SWE and SWP would reduce CH_4 emissions and N excretions and increase milk production without alterations to DMI. This experiment determined that CH_4 emissions were not influenced by SWE or SWP, nor was milk production. Therefore, this portion of the hypothesis is rejected. However, SWP reduced daily UN excretion by 18.1% and SWE tended to reduce daily UN excretion by 14% compared with the control, independently of DMI and at the same N intake. It is therefore concluded that SWP and possibly SWE can reduce UN excretion without negatively affecting DMI and milk production, thereby reducing the environmental impact of herbage based dairy systems.

5.5.1 Dry Matter Intake, methane emissions, and milk yield

The cows during the non-lactating phase were offered 17 kg per d of lucerne baleage, which was unrolled and fed out on the ground. The average DMI across the treatments during the dry period was

14.4 kg/cow per d, so that there was a 15.3% refusal. This is similar to the average refusal (17%) reported by Stockdale (2010). Additionally, the DMD estimated by IADF (average of 64.5%; Table 5.3) is similar to the NIRS predicted DMD (63.4%; Table 5.1) for the lucerne baleage.

The cows were allocated an above ground herbage mass of 30 kg/cow per d, assuming a 66% harvesting efficiency, for a targeted intake of 20 kg of DM/cow per d. Based on the average DMI of 17.4 kg/cow per d, we achieved a 58% forage utilization. The intake estimated in the current experiment is similar to grazing dairy cows on other experiments that estimated intake based on pre- and post-grazing forage masses (Box et al., 2017; Mangwe et al., 2019; Mangwe et al., 2020) and when estimated by DMI required for production (Cavanagh et al., 2008). Dry matter digestibility estimated using the internal marker, IADF, was not different between the treatments and was also similar to the results estimated by the NIRS (Table 5.1).

The gas emissions measured by the AHCS in the current experiment fall within the range reported for Jersey × Friesian dairy cows consuming similar levels of DMI (Cavanagh et al., 2008; Jonker et a., 2020). The similar gas emissions between the treatments further support the non-different intakes and DMD estimated in the current experiment, as DMI is the largest driver of gaseous fermentation products (Charmley et al. 2016; Jonker et al., 2017). This is further corroborated by the current experiment as DMI and CH_4 emissions were positively correlated during both the dry ($r = 0.51$; $P = 0.05$) and lactation ($r = 0.64$; $P = 0.02$) periods.

There were no effects of plant extracts detected for gaseous emissions, while other experiments have shown dramatic reductions in CH_4 emissions when ruminants were fed a red seaweed species (i.e. *Asparagopsis armata*; Roque et al., 2019; Kinley et al., 2020). In the current experiment, we had a relatively low number of animals with adequate visits (>20) to the AHCS with a larger than normal standard error of the mean, which may have contributed to the lack of treatment differences. However, a pre-trial power analysis using the 7% coefficient of variation reported by Beck et al. (2018) determined that 5 animals per treatment would be adequate to detect a 13% treatment difference. Alternatively,

this discrepancy may be due to the different species used in the experiments, which has been shown to influence effects on gaseous emissions *in vitro* (Machado et al., 2014; Maia et al., 2016). The seaweed used in the current experiment —*Ecklonia radiata*— is a brown seaweed, which to our knowledge, the effects on CH_4 emissions is largely unknown. Different species of seaweeds contain different bioactive compounds, for example red seaweeds (e.g. *Asparagopsis*) contain bromoform, which acts as a methane inhibitor. Therefore, it is possible that *Ecklonia radiata* would not reduce CH_4 emissions. Likewise, the SWP treatment, which was composed of *Ecklonia radiata* and various terrestrial plants, did not reduce CH_4 emissions. The *Asparagopsis* species used by Roque et al. (2019) and Kinley et al. (2020) being a red seaweed contains were not extracts, but rather dried, whole plants. Accordingly, the lack of effect on gaseous emissions in the current experiment may be due to an unknown supply of bioactive compounds by the SWE and SWP extracts as well as the extraction process of SWE and SWP, rather than the plants used.

The CH_4 yields observed in the current experiment (18 and 15 g CH_4/kg DMI for the dry and lactation phase, respectively) are lower than others reported in the literature for cattle grazing fresh perennial ryegrass herbage (20.4 g CH_4/kg DMI; Jonker et al., 2017). To our knowledge this is the first experiment which measured methane emissions of dairy cattle during the transition period, thus this discrepancy may be related to physiological changes in the dairy cow. During late gestation, the volume of the rumen decreases due to the size of the fetus and this effect on the rumen may influence ingestive, digestive, and ruminal kinetics (Reynolds et al., 2004). Alterations to the rumen, such as ruminal retention times have been shown to manipulate the CH_4 production (Hammond et al., 2014). While there was no effect of stage (i.e. no-lactating vs lactation periods) on daily CH_4 emissions, due to the lower DMI during the dry period, cows had a greater CH_4 yield (g CH_4/kg DMI) during the non-lactating phase compared with the lactating phase. This might be due to the differences in chemical composition of their diet during the two phases and the potential differences in rumen digesta outflow rate (Hammond et al., 2014). The chemical composition of diets, such as fat (Beck et al., 2018; Beck et al.,

2019) and fiber (e.g. NDF) content (Appuhamy et al., 2016) can have a large impact on methane emissions. Beck et al. (2019) have previously illustrated how reductions in daily CH_4 production seen by whole cottonseed supplementation were related to reductions in ADF digestibility. Thus, the differences in CH_4 yield (g CH_4 per kg DMI) between the non-lactating and lactating measurement periods appear to be related to differences in ADF content of the diets.

While CH_4 was positively related to DMI, CH_4 yield decreased with increasing DMI (r = -0.66; $P <$ 0.01). This relationship has previously been described in the literature. Hammond et al. (2014) determined that as DMI increases, there is a subsequent increase in fractional passage rate (%/hour) of both solids and liquids from the rumen and CH_4 yield decreased with increasing passage rates. Finally, dry matter and energy intake are closely related to productivity of all ruminants. Accordingly, there was a positive correlation between estimated DMI and milk yield (r = 0.50; P = 0.02) and milk protein yield (r = 0.46; P = 0.03). As such, it is not surprising that the extract products did not improve milk production, since there were no improvements in DMI or diet digestibility.

5.5.2 Treatment Discrepancy between dry and lactation period

There was no treatment effect observed over the non-lactating period for the plasma urea concentration in the data presented in the current experiment and accordingly, in the previous report examining the effects of SWE and SWP, there were no observed effects on urine N concentration nor ruminal NH_3 (Chapter 4). However, SWP and SWE had lower plasma urea concentration 3 d post calving, and SWP cows had lower milk urea and calculated UN excretion than the CON during both weeks the cows were observed in milk. This discrepancy between the non-lactating period and the lactation period may be due to differences in the diet fed during those periods. Lucerne baleage was fed solely during the dry period, while fresh perennial ryegrass pasture was grazed from approximately 2 weeks prior to calving through to the end of the experiment fresh perennial ryegrass pasture was grazed. Considering the greater CP requirements of lactating cows compared with non-lactating dairy cows, the greater CP content of the lucerne baleage diet fed over the non-lactating period may explain why the plasma urea

concentration was greater at d 38 and 80 after treatment application compared with d 97 (3 d post-calving; Figure 5.1). Thus, it is believed that this discrepancy is perhaps due to the differences in dose used of SWP and SWE in the non-lactating period (5 mL/cow per d) compared with the lactation period (100 mL).

5.5.3 N partitioning

There was a correlation (r = 0.51; *P* = 0.01) between the plasma urea concentration, measured 3 d post calving, and to milk urea concentrations measured on d 7 of lactation in the current experiment. Urea can be found in blood, milk, and urine of dairy cows and these urea pools have linear relationships (Kauffman and St-Pierre, 2001; Kohn et al., 2002; Kohn et al., 2005). Using these relationships, models have been developed to predict daily UN excretions based on urea concentrations of blood (Kohn et al., 2005) and milk (Kauffman and St-Pierre, 2001; Kohn et al., 2002). In dairy systems, milking typically occurs twice a day, from which milk urea can be tested to provide a convenient means to estimate daily UN excretions. Accordingly, the current experiment employed MUN concentration to estimate daily UN excretions.

The range of daily estimated UN excretion seen in the current experiment is similar to other experiments. Groff and Wu (2005) reported UN excretions between 128.7 to 326.4 g per d from multiparous Holstein dairy cows fed increasing levels of CP and different ratios of lucerne and corn silage. Likewise, the current values are similar to Friesian × Jersey dairy cows grazing perennial ryegrass pasture (Box et al., 2017). The dairy cows provided SWP had 18.1% less predicted urinary N excretion (236.91 g urinary N per d) than the CON (289.14 g Urinary N per d). The mode of action for the reduction in estimated UN excretion by the extracts may be a result of the bioactives contained in the extracts, which have been shown to reduce UN excretions; however, the amount of bioactives provided by the extracts is unclear. Additionally, these products may be altering ruminal fermentation, either through bioactive or probiotic effects, resulting in reduced protein fermentation in the rumen or improving bacterial cell protein production. Both of these means of altering ruminal fermentation would reduce the

amount of ammonia production in the rumen, subsequently decreasing urinary urea production (Bach et al., 2005). Ultimately, the exact mode of action behind these products are unclear and require further investigation.

An 18.1% reduction in daily UN excretion would indicate much less N loading (g N per ha) at the urine patch and total UN deposited onto the paddock, which would subsequently reduce N leaching, or N lost from urine patches as greenhouse gases. As the volume of each urination stays relatively constant due to bladder volume being the physiological stimulant to urinate (Andersson and Arner, 2004; Gregorini et al., 2018), it is safe to assume that the 18.1% reduction in predicted UN excretion would likely translate to a 18.1% reduction in N loading (kg UN/ha) at the urine patch level. Therefore, if we assume that a dairy cow has a typical UN loading rate of 1,000 kg UN/ha (Di and Cameron, 2007), then an 18.1% reduction would equate to a new N loading rate of 819 kg UN/ha. When this new N loading rate is applied to the equation of Di and Cameron (2007) which predicts NO_3^-–N leaching based on N loading rate, then there would be a 21% predicted reduction in N leaching as a result of the SWP treatment.

The reduced daily estimated UN excretion, even with numerically similar and non-statistically different DMI, suggests a greater N use efficiency for the cows offered SWP and a tendency for cows offered SWE. As the amount of milk protein did not increase, this would imply a greater N retention. Accordingly, when apparent N retention was calculated [N intake minus N excretion (from urine, fecal, and milk)], there were numerical differences in the manor that one would expect, but this difference was not significant (Table 5.6). In fact, apparent N retention was highly variable [260% coefficient of variation (**CV)**], and this large variability is likely due to the compounding of measurement errors. As the calculated apparent N retention was dependent on using estimated variables, such as N intake based on inert markers (26.2% CV), calculated UN excretion (30.8% CV), fecal N excretion (34.5% CV), and milk N excretion (19.6% CV), it is not surprising that the CV for apparent N retention was so large. Therefore, while we are unable to conclude that SWE and SWP would result in greater N retention during early

lactation, due to the variability of the data, the large reductions in daily predicted UN excretions, with no differences (numerical or statistical) in N intake or milk and fecal N excretion (g/d), likely indicate less N loss. However, further investigation is required to confirm this.

5.6 Conclusions

Fermented plant extract product based on a brown seaweed and other terrestrial plants (SWP) has the potential to reduce UN excretion (52.2 g UN/d less; 18.1% difference) of dairy cows in early lactation. Further, fermented seaweed extract product (SWE) tends to reduce UN excretion (40.6 g UN/d less; 14% difference), both with no negative effects on DMI or milk production, and without additional inputs, such as grain supplementation. Based on the results of this experiment, we conclude that SWP can reduce UN excretion, thereby reducing the environmental impact associated with grazing based dairy systems.

Table 5.1 Nutritive composition of a ryegrass (Lolium perenne) based pasture and the pelleted supplement used as an attractant for the automatic head chamber system (AHCS) estimated using a calibrated near infrared spectrometer.

Item[a]	Lucerne Bailage[b]	SD	Pasture	SD	AHCS Bait
DM, % as-fed	42.6	2.0	16.9	2.7	90.5
OM, % DM	91.4	0.9	90.5	0.5	94.2
NDF, % DM	41.4	3.8	39.7	1.9	39.1
ADF, % DM	33.2	2.7	22.3	0.9	21.3
CP, % DM	20.7	1.5	16.7	2.7	15.7
WSC, % DM	NA[c]	NA[c]	23.4	4.0	NA[c]
DMD, % DM	63.4	3.4	80.8	1.2	75.9
OMD, % OM	64.9	4.4	87.3	1.4	81.3

[a] DM = dry matter, OM = organic matter, NDF = Neutral detergent fiber, ADF = Acid detergent fiber, CP = crude protein, WSC = water soluble carbohydrates, DMD = DM digestibility, OMD = OM digestibility
[b] Nutritive value has been reported previously in Chapter 4
[c] NA = not available

Table 5.2 Body condition score (BCS) and body weight of grazing lactating dairy cows receiving a daily oral drench of water (CON), a fermented seaweed extract (SWE), or a fermented seaweed plus terrestrial plant extract (SWP).

	Treatments				*P*-value
Item	CON	SWE	SWP	SEM	TRT
n	8	8	7	---	---
BCS	4.38	4.50	4.60	0.14	0.57
Body Weight, kg	494.8	480.6	493.0	18.6	0.79

Table 5.3 Intake and digestibility of non-lactating pregnant (dry) cows fed lucerne bailage and grazing lactating dairy cows grazing a ryegrass dominant sward, receiving a daily oral drench of water (CON), a fermented seaweed extract (SWE), or a fermented seaweed plus terrestrial plant extract (SWP).

	Dry			Lactation				*P*-value[a]		
Item[b]	CON	SWE	SWP	CON	SWE	SWP	SEM	TRT	Stage	Inter.
n	8	8	8	8	8	7	---	---	---	---
Silage/Forage intake	14.0	14.4	14.8	17.2	17.7	17.3	0.94	0.87	<0.01	0.92
AHCS bait intake	0.65	0.39	0.44	0.51	0.51	0.34	0.11	0.20	0.67	0.43
Total intake	14.7	14.8	15.2	17.7	18.2	17.6	0.94	0.93	<0.01	0.88
DMD, %	64.3	64.5	64.8	81.9	81.6	82.7	0.52	0.96	<0.01	0.76
OMD, %	69.0	68.8	69.5	86.4	86.1	87.1	0.60	0.85	<0.01	0.96
NDFD, %	59.7	60.3	61.1	81.4	81.1	82.5	0.83	0.82	<0.01	0.85
ADFD, %	60.8	61.4	61.5	78.8	78.4	79.9	0.85	0.94	<0.01	0.71
CPD, %	75.5	75.1	75.7	78.3	77.1	79.6	0.85	0.21	<0.01	0.54

[a] Analysis of variance *P*-value; TRT = main effect of treatment; Stage = main effect of measurement period (Dry or Lactating); inter. = TRT × Stage interaction.

[b] Silage/Forage intake = intake of silage (dry period) or forage (lactation period), kg/d; GF bait intake = GreenFeed supplement intake, kg/d; Total Intake = total intake, kg/d; DMD = dry matter digestibility; OMD = organic matter digestibility; NDFD = neutral detergent fiber digestibility; ADFD = acid detergent fiber digestibility; CPD = crude protein digestibility.

Table 5.4 Milk yield and composition of grazing lactating dairy cows during the first two weeks of lactation receiving a daily oral drench of water (CON), a fermented seaweed extract (SWE), or a fermented seaweed plus terrestrial plant extract (SWP).

	Treatments				*P*-value[d]
Item	CON	SWE	SWP	SEM	TRT
n	8	8	7	---	---
Milk Yield, kg/d	27.0	27.5	27.4	0.7	0.88
Milk Solids, kg/d[e]	2.4	2.4	2.5	0.06	0.76
% Fat	5.1	4.9	5.4	0.2	0.63
% Protein	3.9	3.9	3.9	0.07	0.44
% Lactose	5.1[a]	5.2[a]	4.9[b]	0.05	<0.01
Fat, kg/d	1.4	1.4	1.5	0.05	0.81
Protein, kg/d	1.0	1.1	1.1	0.03	0.96
Lactose, kg/d	1.4	1.4	1.3	0.06	0.56
Milk Urea N, mg/dL	22.60[a]	19.69[ab]	18.65[b]	1.12	0.04

[a-c] means within a row with different superscripts differ ($P \leq 0.05$).
[d] treatment x day interactions were not significant and are not reported ($P > 0.10$).
[e] Milk solids is fat + protein

Table 5.5 Enteric gas emissions (methane [CH_4] and carbon dioxide [CO_2]) of non-lactating pregnant (dry) fed lucerne bailage and grazing lactating dairy cows receiving a daily oral drench of water (CON), a fermented seaweed extract (SWE), or a fermented seaweed plus terrestrial plant extract (SWP).

	Dry			Lactation				*P*-value[a]		
Item[b]	CON	SWE	SWP	CON	SWE	SWP	SEM	TRT	Stage	Inter.
n	5	5	4	5	4	5	—	—	—	—
CH_4	258	270	285	257	246	243	20.4	0.94	0.21	0.60
CO_2	8,460	8,292	9,419	10,631	9,568	9,377	577	0.59	0.03	0.18
CH_4/CO_2	0.03	0.03	0.03	0.02	0.0	0.02	0.001	0.36	<0.01	0.66
CH_4/DMI	17.3	17.7	19.0	15.6	14.9	14.5	1.42	0.95	0.02	0.62
CH_4/MY	—	—	—	9.3	9.2	9.0	0.4	0.87	—	—
CH_4/MS	—	—	—	103	96	95	3.7	0.25	—	—

[a] Analysis of variance *P*-value; TRT = main effect of treatment; Stage = main effect of measurement period (Dry or Lactating); inter. = TRT x Stage interaction

[b] CH_4 = daily methane production (g/d); CO_2 = daily carbon dioxide production (g/d); CH_4/DMI = methane per kg dry matter intake (g CH_4/ kg DMI); CH_4/MY = methane per kg of milk yield (g CH_4/r kg milk); CH_4/MS = methane per kg of milk solid (g CH_4/kg milk solid).

Table 5.6 Milk urea and milk urea N concentrations used to predict daily urinary N excretion (UN_{pred}) of grazing lactating dairy cows receiving a daily oral drench of water (CON), a fermented seaweed extract (SWE), or a fermented seaweed plus terrestrial plant extract (SWP).

	Treatments				*P*-value[d]
Item	CON	SWE	SWP	SEM	TRT
n	8	8	7	---	---
N Intake, g/d	496.5	485.5	469.6	26.2	0.77
$UN_{pred.}$, g/d[e]	289.1[a]	248.5[ab]	236.9[b]	16.6	0.05
Fecal N excretion, g/d	104.8	111.5	96.2	7.5	0.35
Milk N excretion, g/d	169.9	167.6	169.0	6.9	0.97
Apparent N retention, g/d	-61.6	-43.0	-33.5	25.6	0.74

[a-c] means within a row with different superscripts differ ($P \leq 0.05$).

[d] All interactions (treatment × day; treatment × time; day × time; treatment × day × time) were not significant ($P > 0.10$).

[e] Predicted daily Urinary N excretion based on the equation proposed by Kohn et al. (2002):
UN (g/d) = 0.0259 × Body Weight (kg) × Milk Urea Nitrogen (mg/dL).

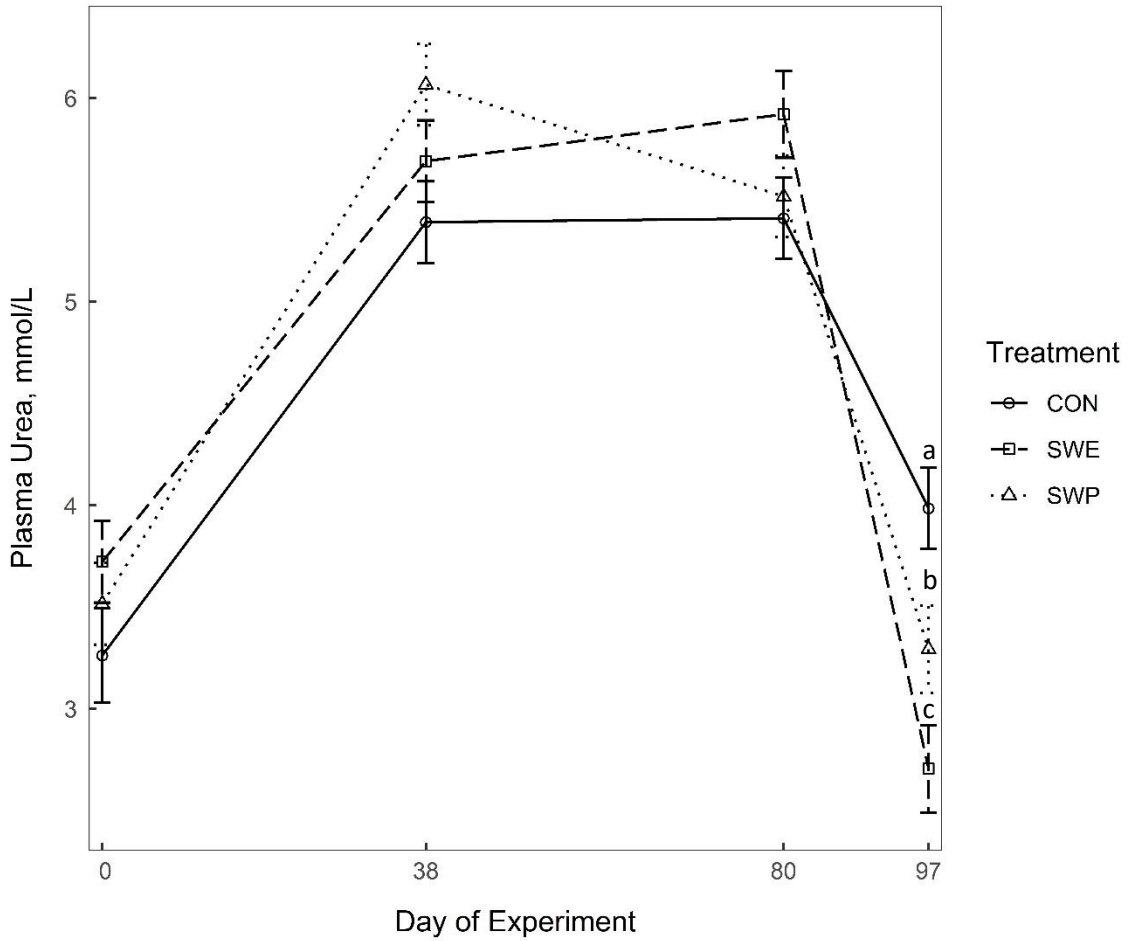

Figure 5.1 Plasma urea concentrations of grazing lactating dairy cows receiving a daily oral drench of water (CON), a fermented seaweed extract (SWE), or a fermented seaweed plus terrestrial plant extract (SWP). Blood samples were taken prior to treatment application, 38 d, 80 d, and 97 d, which is three days after calving, after applying the treatments.

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Chapter 6

Negative effects of energy supplementation at peak lactation of sheep can be offset by the addition of fermented plant extracts

6.1 Abstract

The current experiment examined if a pelleted energy supplement with or without the addition of *Lactobacillus* fermented seaweed or seaweed plus terrestrial plants extracts impacted oxidative stress of ewes from late gestation through to weaning and ewe and lamb production from lambing to weaning. Treatments were either no supplement (CON-), a pelleted supplement only (CON+, 100 g/ewe per d), CON+ with seaweed extract only (SWO, 10-mL/ewe per d), or CON+ with seaweed plus an arrangement of terrestrial plant extract (SWP, 10-mL/ewe per d). Ewes (n = 160; mean initial BW = 72.3 ± 9.5 kg [mean ± standard deviation]) were randomized to pastures (n = 4 pastures per treatment with 10 ewes each). After lambing, ewes with twins were reallocated to pastures (n = 3 pastures per treatment with 10 ewes each) according to lambing date. At weaning, supplementation tended (P = 0.10) to reduce total antioxidant status (TAS) and increased (P = 0.04) glutathione peroxidase (GPx) activity compared with non-supplemented ewes. The addition of seaweed and terrestrial plants extracts to the concentrate, i.e. SWO and SWP, increased (P < 0.05) TAS and reduced GPx activity compared with CON+. Supplementation increased (P < 0.05) milk yield at wk 4, 6, and 8 of lactation, and protein, lactose, and total milk solids yield at peak lactation (wk 4). The CON- ewes had greater (P = 0.03) somatic cell count than the supplemented ewes at week 4, 8, and 10 of lactation. Our results suggest energy supplementation, alone, increases oxidative stress of lactating ewes, which may relate to increased oxidative phosphorylation. Most importantly, these results indicate that in situations where energy supplementation is needed to increase animal performance, negative effects of energy supplementation

around peak lactation can be offset by the addition of *Lactobacillus* fermented plant extracts (SWO and SWP) to improve antioxidant status.

Keywords: grazing; sheep milk production; oxidative stress; probiotics

6.2 Introduction

Oxidative stress, is defined as the imbalance between oxidants (e.g. superoxide anion) and antioxidants [both enzymatic (e.g. superoxide dismutase) and non-enzymatic (e.g. glutathione)] (Sordillo and Aitken, 2009). Several stressors have been shown to increase oxidative stress and thereby increase the incidence of morbidity and mortality in livestock (Chirase et al., 2004; Sordillo and Aitken, 2009; Celi, 2010). For example, handling stress (Fidan et al., 2010) and shearing (Fidan et al., 2009) have led to increased blood cortisol (indicating physiological stress), a greater concentration of plasma malondialdehyde (MDA; a marker for oxidative stress), and less glutathione (as a marker of antioxidant status). Transport stress in cattle caused an increase in MDA and reduced total antioxidant capacity in serum (Chirase et al., 2004). Following transportation, calves that required ≥3 treatments for bovine respiratory disease, throughout a finishing phase, had 2-times greater MDA concentrations than their healthier cohorts at arrival (Chirase et al., 2004). Specific to mature breeding and lactating livestock, unfulfilled energy demand is a significant source of stress (Sordillo and Aitken, 2009; Celi, 2010; Singh et al., 2011). Such a negative energy balance causes nutritional stress, which in turn produces reactive oxygen metabolites and subsequently oxidative stress, reducing health and welfare of the lactating animals by enabling disorders like mastitis, metritis, and retained placenta (Sordillo and Aitken, 2009; Celi, 2010).

One possible means to reduce oxidative stress in transition and lactating grazing animals is to reduce the negative energy balance by strategic supplementation of the base forage with energy rich concentrates (Sordillo et al., 2009). However, energy supplementation through starch may also increase oxidative stress by increasing oxidative phosphorylation, especially around peak lactation (around 80 days in milk in dairy cows, i.e. peak lactation) (Gabai et al., 2004). Another alternative to reduce oxidative stress is feeding plant extracts, such as seaweed products (Beck and Gregorini, 2020). For example,

Kannan et al. (2007) found that the addition of a seaweed extract to the diet of transport stressed goats improved antioxidant status. Additionally, several experiments determined that probiotic supplements based on *Lactobacillus* species can reduce oxidative stress in ruminants (Ran et al., 2019; Izuddin et al., 2020). Therefore, it was hypothesized that energy supplementation of the base forage diet of lactating grazing ewes would reduce oxidative stress and that this effect would be enhanced by the supplemental addition of *Lactobacillus* fermented extract products based on seaweed (Animal Health Tonic) and seaweed plus terrestrial plants (Fortress+; AgriSea Ltd., Paeroa, New Zealand). The objective of the current experiment was to determine the effect of a pelleted energy supplement with or without the addition of a seaweed or seaweed plus terrestrial plants extracts on oxidative stress and milk production and composition of ewes from late gestation through to weaning.

6.3 Materials and Methods

This experiment took place at the Lincoln University sheep farm from 8 July to 14 December 2018. All procedures outlined here were approved by the Lincoln University Animal Ethics Committee (AEC 2018-25).

6.3.1 Description of Products

The current experiment was designed to explore the health, in terms of ameliorating oxidative stress, and rumen fermentation effects of two *Lactobacillus* fermented plant products. One of the products is based on the fermentation of a brown seaweed (Ecklonia radiata) (SWO; Animal Health Tonic, AgriSea New Zealand Seaweed Ltd.; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand). The other product is based on the fermentation of seaweed, plantain (*Plantago lanceolota*), chicory (*Chicorium intybus*), broad-leaf dock (*Rumex obstusifolius*), and lucerne (*Medicago sativa*) (SWP; Fortress+; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand). Both extract products followed the extraction process, which was based on a fermentative extraction technique of the seaweed, in the case of SWO, as well as the seaweed and the terrestrial plants for SWP according to proprietary methods

(Bradley; AgriSea New Zealand Seaweed Ltd.). These extracts are thought to provide health benefits through providing Lactobacillus fermentation metabolites of compounds extracted from the plant species, prebiotics, and probiotics.

Prior to the start of this experiment, the antioxidant capacity of SWO and SWP were investigated using the stable free-radical compound, 2,2-diphenyl-1-picryl-hyrazyl-hydrate (DPPH), which is purple in color while in the free-radical form. In the presence of antioxidants, i.e., free-radical scavengers, the radical is scavenged and DPPH goes from purple to clear in color. Thus, the inhibition of DPPH is measured colorimetrically by measuring changes in absorption at 515 nm (Olejar et al. 2019). Using the methods described by Olejar et al. (Olejar et al. 2019), we determined that SWO and SWP had a 33.9% ± 1.4 and 48.7% ± 1.2 (mean ± standard deviation) inhibition, respectively. These levels of DPPH inhibition are similar to values reported for methanolic extracts of different brown seaweed species [*Ascophyllum nodosum* (25.6%) and *Fucus vesiculosus* (31.2%); O'Sullivan *et al.* 2011).

In order to determine the specific *Lactobacillus* species, 1 mL of SWO and SWP products were aseptically transferred into 9 mL of buffered peptone water and proceeded with the serial dilution procedure. The serial dilutions of the samples (10^{-1}, 10^{-2}) were prepared and 1mL of the sample from 10^{-2} dilutions were pipetted using a sterile pipette and aseptically transferred to specialized culture media, MRS agar [MRS agar-code 1219 (Fort Richard); used for isolation of *Lactobacillus* species]. The predominant species were determined as *Lactobaccillus paracasei*, using 16S ribosomal RNA sequencing (Lane et al., 1985) using universal primers.

6.3.2 Animals and Treatments

Coopworth ewes at the Lincoln University Sheep Farm were ultra-sound scanned to determine if they were pregnant with a single or multiple lambs. Based on these results, ewes carrying multiple offspring (n = 160; body weight = 72.3 ± 9.5 kg; 3.8 ± 1.2 years old; 2.8 ± 1.2 parturitions [mean ± standard deviation]) were randomly assigned to one of four dietary treatments: 1) negative control, which received no supplement (CON-); 2) positive control, which was offered the basal supplement only

(CON+); 3) the basal supplement with 10-mL per ewe per d of SWO; or 4) the basal supplement plus SWP at 10-mL per ewe and d. The basal supplement was a commercially available pellet labeled to supply 12.2 MJ of metabolizable energy/kg dry matter (DM), 12.2% crude protein (CP), and 2% fat (Sheep Nuts, Reliance Feeds, Canterbury, New Zealand). Addition of the SWO and SWP extracts occurred by adding 2.5-L of the respective extract products to 25-kg of pelleted supplement, mixed in a concrete mixer for 5-min, and then were spread on a concrete pad for 2-days to dry. The proportion of ewes, excluding CON-, that did not approach the feed bunk when supplements were provided averaged 17.5 ± 10.5% (mean ± standard deviation) and was not affected by treatment. The refusal to consume supplements is common in grazing ruminants and the values seen in the current experiment are similar to those reported by others (Dixon et al., 2003).

After being randomized to treatments, ewes were randomly allocated to spatially different pastures (replicates, four per treatment) of the same vegetative sward (approximately 60% Ryegrass [*Lolium perenne* L.], 30% white-clover [*Trifolium repens* L.], and 10% weeds), with each pasture containing ten ewes per pasture. Ewes remained on their pastures until after lambing After lambing, ewes with twins were reallocated to pastures (n = 3 pastures per treatment with 10 ewes each) according to lambing date. Additionally, pastures were grouped by lambing date so that the twin bearing ewes were placed into pastures with similar aged lambs. Only the pastures with twins were used for data collection in order to control for any potential effects associated with the number of lambs being raised by the ewes. All pastures were grazed using continuous stocking as the grazing management. Stocking rate was the same at 10 ewes in a 0.75-ha pasture during the whole experimental period. Herbage mass was determined thrice, 29 September, 6 November and 16 December, using the calibrated rising plate meter (Jenquip; Feilding, New Zealand), with one equation being established for all pastures. Calibration equations were developed by taking plate readings at 10 locations with a continuum of compressed herbage heights to encompass the herbage mass present in the pastures and then clipping the herbage at the 10 locations to ground level. The clipped herbage was weighed fresh and a subsample was oven-

dried at 60 °C to determine DM percentage. The DM% was used to calculate total DM of herbage in the clipped area, which was then extrapolated to a kg DM per ha basis. These values were fitted with a regression equation along with the plate meter compressed heights readings to develop the calibration equation (R^2 = 0.94). Herbage mass throughout the experiment averaged 2,229 ± 456 kg DM/ ha and provided 1.72 ± 0.4 kg of herbage DM/ kg of body weight. A subsample of herbage clippings was freeze-dried, ground, and analyzed for chemical composition by near-infrared spectroscopy (NIRs), using pre-established equations. Nutritive values used for the calibration of NIRs were obtained for DM and ash (AOAC, 1990; method 930.15 and 942.05, respectively), NDF (Van Soest et al., 1991) and ADF (AOAC, 1990; method 973.18), CP by combustion (Variomax CN Analyser Elementar), water soluble carbohydrates (MAFF, 1986), and DM digestibility, OM digestibility, and digestible OM in the DM (DOMD) (Iowerth et al., 1975). All calibration equations had $R^2 \geq 0.90$, and NIRs readings for the forage samples were within the calibration range. The metabolizable energy (ME) content of the herbage provided was then calculated as 0.16 multiplied by DOMD content (Primary Industries Standing Committee, 2007). The herbage was determined to contain (mean ± standard deviation) 30.6 ± 7.5% DM, 90.2 ± 2.8% OM, 46.3 ± 3.1% NDF, 25.9 ± 1.8% ADF, 11.0 ± 1.8% CP, 23.6 ± 0.6% water soluble carbohydrates, 72.3 ± 3.8% DM digestibility, 77.5 ± 4.2% OM digestibility, and 10.5 ± 0.6 MJ ME/kg DM.

6.3.3 Data and Sample Collection

Ewes were weighed unfasted at trial initiation (8 July 2018) and weaning (14 December 2018). During lambing (from 26 August 2018 to 18 September, 2018), pastures were checked daily (0800 AM) and any new lamb was weighed with birth weight, sex, and date of birth recorded and allocated an electronic identification (EID) ear tag. Lambs were also weighed at tailing (9 October 2018) and weaning (14 December 2018) to calculate average daily gain (ADG) during different periods up to weaning.

Samples were collected from focal ewes (n = 5 per pasture) which were selected at randomly. The same focal animals were sampled for blood and fecal samples prior to lambing, 4 weeks after lambing, and at weaning and for ruminal fluid at weaning. Blood samples were collected (10 mL heparinized blood tube)

by jugular venipuncture. Two mL of heparinized whole blood was removed from the blood tubes, placed into a 2-mL Eppendorf tube, and stored at -20ºC until analysis. Blood tubes were then centrifuged at 2300×g for 10 min at 4ºC and plasma was aspirated and stored at -20ºC until analysis. Fecal samples were collected by rectal grab, lyophilized and then ground by a centrifugal mill (ZM200 Retsch) to pass through a 1 mm screen. Ruminal fluid was obtained from by esophageal tubing. Two, 2 mL subsamples of the ruminal fluid were immediately placed into tubes, one containing sulfuric acid (to inhibit NH_3 volatilization) for the determination of NH_3 and one without for the measurement of volatile fatty acids (VFA) concentration.

Milk production was measured from two pastures per treatment containing the earliest lambed ewes. The first group of measured pastures lambed on 3 September 2018 ± 3.8 d and the second on 11 September 2018 ± 3.0 d, with one pasture per treatment in each group. Milking of the first group began on 2 October 2018 and the second group on 9 October 2018, so that milking began approximately 4-wk post-lambing. Each group of sheep in assigned pastures were milk sampled every-other week, until 20 November 2018. Milking was done as previously described by (Yusuf et al., 2018), implementing the four hour milking interval technique (Hunter et al., 2015). Ewes were brought from pastures to the sheep yards and lambs were separated from ewes. Ewes were milked using a portable milker (Type: DVP170/340; DeLaval; Tumba, Sweden) to remove existing milk from the udder and were then held for four hours. The ewes were then administered an intramuscular dose of oxytocin (1.0 mL, 0.0167 mg/mL, 10 IU per mL, Kela Health, Hoogstraten, Belgium). After 1-min ewes were milked again using the same portable milking system, but this time fitted with sub-sampling containers containing bronopol (0.1%). Samples were stored at 4°C until analysis, which was done within four days of collection.

6.3.4 Sample Analysis

Fecal samples were analyzed for nitrogen (N) by combustion (Variomax CN Analyser, Elementar Analysensysteme, Germany). Non-acidified ruminal fluid was analyzed for VFA concentration using gas chromatography (GC-2010, Shimadzu, Kyoto, Japan) fit with a SGE BP21 30 m x 530 µm x 1.0 µm bore

capillary column. The acidified ruminal fluid sample was analyzed for NH_3 concentration by an enzymatic UV method (Neeley and Phillipson, 1988) using an automated clinical analyzer (Randox Rx Daytona, Crumlin, County Antrim, UK). Heparinized whole blood samples were analyzed for the antioxidant enzyme, glutathione peroxidase (GPx), according to the Randox kit manual (RANSEL; catalog number RS504), which is an enzymatic based protocol, and was analyzed using an automated clinical analyzer (Randox Rx Daytona, Crumlin, County Antrim, UK). Plasma samples were analyzed for total antioxidant status (TAS) using a colorimetric method and for urea N (PUN) using a commercial enzymatic kinetic technique according to their respective manuals (Randox, Crumlin, County Antrim, UK). These assays were both analyzed using the Randox Rx Daytona (Crumlin, County, Antrim, UK).

Milk samples were analyzed for fat, protein, lactose, and somatic cell count (SCC) by a MilkoScan (Foss Electric, Hillerød, Denmark), which was calibrated for sheep milk samples. Based on the quantity of milk sampled by the portable milker sub-sampling unit, the total amount of milk produced during the four hours between the two milkings were calculated according to the calculations provided by Yusuf et al. (2018). Milk yield data is reported as kg produced per 4-hr.

6.3.5 Statistical Analysis

This experiment was a completely randomized design. Pasture was the experimental unit and individual sheep was used as a random effect. All data were fit by a mixed effects model using the 'nlme' package (Pinheiro et al., 2018). Data from milk, blood, and feces included sampling time, treatment, and the sampling time-by-treatment interaction as fixed effects in the model. The data available which were not repeated measures, including ewe initial and final body weight, ewe weight change, lamb birth, tailing, and weaning weights, and ruminal samples only included treatment as a fixed effect.

Milk composition (somatic cell count and fat, protein, lactose, Solids no fat [Lactose + protein], and milk solids [MS; fat, protein, and lactose] percent) were fit by a generalized linear model with the 'glmer' function from the 'lme4' package (Bates et al., 2015). The model included fixed effects for treatment, the week post-lambing, and the treatment by week post-lambing interaction. Random effects

were milking groups and individual animal. For the percentage results (fat, protein, lactose, solids no fat, and milk solids) a gamma distribution was used and a Poisson distribution was used for somatic cell count. Milk yield data (fat, protein, lactose, Solids no fat [Lactose + protein], and milk solids [MS; fat, protein, and lactose] yield; kg/4-hr) were fit by a linear mixed effects model using 'lmer' function of the 'lme4' package (Bates et al., 2015). The results from the 'glmer' output were then back-transformed to the response level and means were generated using the 'emmeans' function and these are the values reported (Lenth, 2018).

Finally, treatment effects were compared using pre-planned orthogonal contrasts. The planned contrasts were designed to test: 1) differences between extracts (SWP vs SWO), 2) effect of supplementation (CON- vs CON+, SWO and SWP), and 3) effect of extracts (CON+ vs SWO and SWP). All contrasts were generated using the 'emmeans' package (Lenth, 2018). Additionally, Pearson's correlation coefficients were generated using the 'cor.test' function for some of the variables to assist with discussion. Significance was declared at $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.1$. All statistical analysis was conducted using R (R Core Team, 2020) (v.3.4.4).

6.4 Results

6.4.1 Ewe and Lamb Body Weights

There were no effects for initial or final body weight for any of the generated contrasts ($P \geq 0.16$; Table 6.1). Supplementation did not affect ewes body weight change (i.e. CON- vs Supplemented; $P \geq 0.28$; Table 6.1). However, SWP lost less weight, from trial start to weaning, than SWO (i.e. SWO vs SWP; $P = 0.04$). Treatment did not affect lamb birth, tailing, or weaning weights ($P \geq 0.14$; Table 6.2). There was no treatment effect on lamb ADG from birth to weaning ($P \geq 0.25$; Table 6.2).

6.4.2 Blood Analysis

Plasma urea concentration of ewes was not different at the pre or post-lambing sampling dates ($P \geq 0.17$). At weaning, supplementation increased plasma urea compared with the non-supplemented ewes ($P < 0.01$). Although, at weaning there was no effect of treatment on plasma urea concentrations when comparing both SWO and SWP to the CON+ treatment ($P = 0.75$), though SWP plasma urea concentrations

was lower than SWO (P = 0.05; Table 6.3). Treatment did not affect total antioxidant status either prior to lambing or at weaning ($P \geq 0.22$). Four weeks after lambing, CON- ewes tended to have greater TAS than the supplemented treatments (P = 0.10). Post-lambing, CON+ ewes had lower (11%) TAS than the ewes fed SWO and SWP (P = 0.04; Table 6.3). Before lambing, there was no treatment effects on GPx activity in the whole blood ($P \geq 0.53$). However, GPx activity was lower for CON- ($P < 0.01$) ewes than ewes fed supplement ($P < 0.01$) and lower for the ewes provided SWO and SWP compared to CON+ (P = 0.05).

6.4.3 Milk Composition

Milk constituents over the milking period (weeks 4-10 post-lambing) are shown in Figure 6.1. Milksolids percent (fat, protein, and lactose percent) were not different ($P > 0.10$) between treatments until week ten after lambing. Ten weeks after lambing, SWP had less (P = 0.02) milksolids proportion than SWO, and CON+ greater than SWO and SWP (P = 0.02). The differences seen in milksolids at week ten were largely due to difference in fat content, as SWP had lower fat content than SWO ($P < 0.01$). Additionally, four weeks after lambing SWO and SWP ewes had lower (P = 0.05) fat concentration than CON+. Protein and lactose concentration were also only different at week ten after lambing, with CON- ewes producing less than ewes fed supplements (P = 0.05). In addition, ten weeks after lambing, SWO and SWP treatments reduced milk protein concentration compared with CON+ (P = 0.03). Ewes in CON- produced milk with lower lactose concentration ($P \leq 0.03$) compared with the ewes fed supplements at weeks four and ten after lambing. Ewes under SWO and SWP treatments had greater milk lactose concentration at week eight compared with CON+ ($P < 0.01$). Ewes receiving supplement had lower SCC than CON- at weeks 4, 8, and 10 post-lambing ($P \leq 0.03$). Additionally, CON+ had lower SCC than ewes under SWO and SWP at week 4 of lactation (P = 0.03).

Milk yield results are displayed in Figure 6.2. Supplementation increased ($P < 0.05$) milk yield (kg/4-hr) during week four (+20%), 6 (+22%), and 8 (+50%) of lactation. The only treatment specific effects for fat yield, protein yield, lactose yield, and total milksolids yield were observed at 4-wk of lactation. The sheep provided the plant extracts (i.e. SWO and SWP) had less ($P < 0.05$) milksolids and fat

yield than CON+. Additionally, the supplemented treatments (i.e. SWO, SWP, and CON+) had greater (P < 0.05) protein yield, lactose yield, and milk solids yield than the non-supplemented ewes. Milksolids (%) and milk yield were negatively correlated (r = -0.25; P < 0.01). Milk yield was not correlated to TAS (r = -0.10; P = 0.56) or GPx (r = -0.03; P = 0.85).

6.4.4 Ruminal Fluid Parameters

Supplementation increased (P < 0.01) ruminal NH_3 concentrations at weaning (Table 6.4). The SWO extract increased acetate concentration compared with SWP (P = 0.04), but there were no other treatment differences detected for acetate. There were no treatment specific effects on propionate concentration ($P \geq 0.13$). Ewes receiving supplement had greater butyrate concentration compared with CON- (P = 0.04), and ewes under SWO had greater butyrate concentration than SWP (P = 0.02). Ewes under CON- had lower valerate, iso-butyrate, and iso-valerate concentrations than the ewes receiving supplement ($P \leq 0.02$). Supplementation tended to reduce the acetate: propionate ratio compared with CON- (P =0.07), and SWP had a lower acetate: propionate ratio compared with SWO (P = 0.02). There was no treatment effect on total VFA concentration detected from supplemented ewes compared with CON- (P = 0.44) nor from the addition of the extracts compared with CON+ (P = 0.59); however, SWO tended (P = 0.06) to have greater total VFA concentration than SWP (Table 6.4). Ruminal NH_3 was positively correlated with iso-butyrate (r = 0.82; P < 0.01) and iso-valerate (r = 0.85; P < 0.01). Plasma urea concentration was positively correlated with NH_3 (r = 0.64; P < 0.01), iso-butyrate (r = 0.60; P < 0.01), and iso-valerate (r = 0.61; P < 0.01).

6.5 Discussion

It was hypothesized that energy supplementation to the dietary base, i.e. grazed herbage by ewes would help account for the negative energy balance experienced during lactation; and that, such an energy supplementation fed to ewes would cause glucogenic shifts of ruminal fermentation patterns increasing energy supply and thereby ameliorating oxidative stress. Our results indicate that the energy supplementation alters fermentation patterns at weaning by increasing butyrate, valerate, iso-butyrate,

and iso-valerate concentrations, increasing ruminal NH_3 and tending to reduce the acetate-to-propionate ratio, but increasing oxidative stress at peak lactation. Therefore, the hypothesis was rejected. An additional hypothesis was that oxidative stress is alleviated through the addition of plant extracts (SWO and SWP). The results of the current study support this hypothesis.

6.5.1 Oxidative Stress

Energy supplementation increased oxidative stress 4-wk post lambing, at peak lactation for lactating ewes (Cardellino and Benson, 2002). This is evidenced by the tendency for CON- to have greater TAS and significantly less GPx activity compared with CON+, SWO, and SWP. Total antioxidant status is a biological marker that attempts to measure the overall antioxidant capacity in biological samples and likely provides a better estimate of antioxidant status of animals than simply quantifying individual antioxidants (Ghiselli et al., 2000). Additionally, measurements of antioxidant enzymes, such as GPx, provides estimates of oxidative stress (Celi, 2011). Thus, GPx activity has been used to measure oxidative stress in livestock. Bernabucci et al. (2002) determined increased GPx activity and malondialdehyde (product of lipid peroxidation) in dairy cows transitioning from dry to lactating in the summer than in the spring, indicating that heat stress induces oxidative stress. Therefore, when TAS and GPx results from the current experiment are taken together, energy supplementation increased GPx activity and reduced plasma TAS, but this effect of supplementation was alleviated by the plant extracts (SWO and SWP).

The current results contrast with several reports. Sgorlon et al. (2008) was unable to detect any differences in GPx activity in ewes fed a high starch diet (28% starch in the diet) compared with their control counterparts 35 or 50-d post-lambing. Additionally, a lower density energy diet (5.6% corn) fed to goats resulted in a lower antioxidant capacity at parturition compared with a higher energy diet (19.7 % corn; Celi et al., 2010). However, the differences between these experiments and the current one could be explained by the physiological stage of the animals or due to differences in milk yield. Several works have linked milk production and oxidative stress (Castillo et al., 2003; Gabai et al., 2004; Lohrke et

al., 2004). Castillo et al. (2003) determined that dairy cows at peak lactation (35 L/cow and d) had greater plasma hydroperoxide (measurement of oxidative stress) than cows at sixth month of lactation (20 L/cow and d). This agrees with Lohrke et al. (2004), who found a strong positive correlation (r = 0.86) between daily milk yield and plasma hydroperoxides in dairy cows. However, in the current experiment, we found no correlation between milk yield and TAS or GPx at 4-weeks of lactation, indicating that the increased oxidative stress associated with the starch supplementation may be due to another cause.

Another possibility to explain these results is potential changes in metabolism associated with the different diets given to the sheep. Wullepit et al. (2009) determined that a high energy diet (providing 8 kg of concentrate per d) compared with a lower energy diet (providing 3 kg of concentrate per d) fed to dairy heifers resulted in a lower ferric-reducing ability of plasma, which indicates reduced antioxidant status in the plasma. However, the remaining oxidative stress markers measured were inconclusive (Wullepit et al., 2009). Further, dairy cows provided diets consisting of 20% wheat, had lower TAS and increased superoxide dismutase and GPx activity than those provided 10% or 0% wheat (Guo et al., 2013). When growing lambs were fed different diets containing 83, 52, or 0% maize in the diet, the lambs provided grain had greater MDA concentrations in erythrocytes (Singh et al., 2011). Additionally, Gabai et al. (2004) found that feeding starch based concentrates to lactating dairy cows increased oxidative stress, as evidenced by the greater (20% increase) MDA levels at d 80 of lactation. Peak lactation for dairy cows generally occurs around d 80 of lactation (Hutjens, 2011) and would thus be a comparable point in the lactation curve to the current experiment, as 4-weeks in milk will be near peak lactation for lactating ewes (Cardellino and Benson, 2002). Such a negative effect of a starch-based energy concentrate has been explained by changes related to oxidative phosphorylation (Gabai et al., 2004). Oxidative phosphorylation occurs in the mitochondria and is a major source of oxidants (namely superoxide anion) in mammals (Murphy, 2009). The majority of these oxidants are generated by complex I of the electron transport chain (Murphy, 2009). In ruminants, acetate is the predominant source of energy and after conversion to acetyl-CoA, it can be converted into ketone bodies, which can be used for

energy by the peripheral tissues, or enter into the Krebs cycle through condensation with oxaloacetate, which requires propionate, glucogenic amino acids, lactate, or glycerol to generate. In the events where the acetate-to-propionate ratios are high, acetate will be metabolized at greater rates through conversion to ketone bodies and utilization by peripheral tissues. However, when propionate production is at a rate where gluconeogenesis needs are met, a greater proportion of acetate will enter into the Krebs cycle, thus causing increases in normal oxidative metabolism, through oxidative phosphorylation (Van Soest, 1994). Thus, the increased oxidative stress in our ewes consuming supplement could be attributed to changes in metabolic processes, such as increased oxidative phosphorylation. We speculate that when the oxidants produced from increased oxidative phosphorylation were paired with increased energy demand at peak lactation, the production of oxidants outpaced the antioxidant defense, thus resulting in oxidative stress.

While feeding energy concentrate to the ewes increased oxidative stress, the additional plant extracts alleviated this stress. The SWO and SWP extracts are fermented extract products and therefore contain natural antioxidants from the extracted plants (i.e. seaweed and/or the terrestrial plants), but also lactic acid producing bacteria and *Lactobacillus* fermentation products (metabolites from microbial fermentation). Plant extracts can provide potent antioxidants due to their plant secondary compounds (PSC; namely mono- and poly-phenols; Rice-Evans et al., 1997). There is evidence to support several modes of actions for PSC as antioxidants. These include by reducing oxidants in the gastrointestinal tract, directly reducing oxidants at the tissue level, and by regulating gene expression (namely nuclear factor erythroid 2-related factor 2; Beck and Gregorini, 2020). Moreover, lactobacillus fermentation products can exhibit antioxidant activities. When finishing steers in a feedlot system were provided with *Lactobacillus* fermentation products (at a similar dose to the one used here), there was an increased blood antioxidant status and reduced GPx activity (Ran et al., 2019). Thus, it is proposed that the increased antioxidant status provided by the plant extracts (SWO and SWP) in the current study may provide health benefits through a combination of PSC and lactobacillus fermentation products.

6.5.2 Ruminal Fluid and Plasma Urea

Supplementation altered ruminal fermentation patterns at weaning. These differences were namely a tendency for reduced acetate-to-propionate ratio, and increased butyrate, valerate, the branch chain VFA, and ruminal NH_3 concentrations. A reduced acetate-to-propionate ratio is expected with increased starch intake as propionate production is increased from starch fermentation in the rumen (Orskov, 1986). Increased ruminal NH_3 concentrations in the rumen is less expected, on one hand, as typically starch feeding would namely: 1) reduce daily N intake (Castillo et al., 2001) and 2) increase the proportion of N consumed captured as microbial cell protein (i.e. increase the efficiency of N use) (Bach et al., 2005). On the other hand, there are measurements at weaning that support this finding, particularly the elevated iso-butyrate and iso-valerate (branch chained VFA) concentrations in the ruminal fluid. These are a product of ruminal fermentation of branch-chained amino acids, and subsequently, they have been suggested as markers for ruminal fermentation of amino acids, which would explain the increased ruminal NH_3 concentrations (Apajalahti et al., 2019). This relationship between ruminal NH_3 and branch chained VFA was observed in this current study, as the correlation between NH_3 and iso-butyrate and iso-valerate across animals where strong ($r = 0.82$ and 0.85, respectively). Additionally, plasma urea N concentration was increased at weaning for the supplemented ewes. Following absorption through the ruminal wall, NH_3 is rapidly converted in the liver to urea and either recycled to the rumen, or excreted through the urine. As such, urea in the blood is a strong indicator of rumen fermentation of amino acids (Bach et al., 2005). Consequently, plasma urea concentrations were positively correlated to ruminal NH_3 ($r = 0.64$), iso-butyrate ($r = 0.60$), and iso-valerate ($r = 0.61$) concentrations. Therefore, it appears the supplemented ewes had greater ruminal protein degradation at weaning than the un-supplemented ewes.

While unexpected, further investigation into the literature provides a possible explanation for the starch supplements increasing protein degradation in the rumen at weaning (Back et al., 2005). This

is by potential differences in microbial populations between the supplemented and non-supplemented treatments. By applying the liquid extracts to the supplement, this may alter the bacterial profile of the feed, which may influence the results determined in this experiment. Additionally, supplemental starch may result in shifts of microbial populations to greater amounts of starch fermenting bacteria which would mean greater amylase activity in the rumen (Nozière and Michalet-Doreau, 1997). Amylase has been shown to increase protein degradation by several experiments (see Bach et al., 2005). This is due to the inability of protein degrading bacteria containing proteolytic enzymes to attach to carbohydrate bound proteins (Bach et al., 2005). However, independently of these possible explanations, plasma urea was not elevated at any of the other time points sampled, which may suggest that these results are an artifact of the sampling time, and not necessarily a result from the starch supplement.

6.5.3 Ewe Performance

Ewes under CON- had two incidences of high SCC (>300 × 10^3 SCC/mL; weeks 4 and 8). Additionally, the SWP and SWO treatments had greater SCC than the CON+ treatment at week 4 of lactation (Figure 6.1). This greater SCC for CON- was unexpected, since increments in SCC are indicators of mastitis, which is associated with increased inflammation, and thus, oxidative stress (Harmon, 1994). Moreover, the current results indicated that CON- ewes had greater TAS and lower GPx activity, yet shown several incidence of high levels of SCC. Despite such a disagreement, some reports support the current findings. Feeding lactating dairy cows pomegranate pulp silage (source of phenolic compounds as natural antioxidants) improved antioxidant status with no subsequent improvement in SCC of milk (Kotsampasi et al., 2017). The authors speculate that the current results are related to the relatively low SCC observed in all of our treatments, even the CON-. A SCC greater than 400x10^3/mL of milk is the threshold for subclinical mastitis in lactating ewes (Kern et al., 2013; Zafalon et al., 2016; Persson et al., 2017). There was only one instance where this was exceeded which was CON- at week 8 of lactation (437.5 ± 105.3 × 10^3 SCC; mean ± standard error). All other SCC were below this threshold (Figure 6.1).

Additionally, at week 4, when the blood samples were collected, the SCC of CON- was less than the suggested threshold (332.3 ± 79.8 × 10^3 SCC; mean ± standard error) and much less for the ewes provided the extract products (≤ 214.2 ± 47.8 × 10^3; mean ± standard error). Therefore, we speculate that the level of inflammation occurring in the mammary glands of the sheep was not great enough to disrupt the homeostatic balance of oxidants and antioxidants in the blood of the sheep. It is recommended that further research is needed to confirm these results.

There were some observed differences in milk composition, especially at week 10 of lactation. Overall, the trend of the milk composition data followed similar patterns to those in the literature. Generally, as milk yield (kg of milk per d) decreases throughout lactation, the corresponding concentration of milk solids will increase (Sakul and Boylan, 1992). What was less expected was the differences in milk solids at week 10 of lactation between SWP and the other treatments. This may be due to the numerically greater milk yields for SWP compared with the other treatments at week 10 (Figure 6.2), as there was a moderate negative correlation detected between milk solid (%) and milk yield (kg/4-hr; r = -0.25) across all the animals and milking days.

6.6 Conclusions

Energy supplement feeding increased oxidative stress compared with non-supplemented lactating ewes 4-weeks post-partum, corresponding to peak lactation. Such a stress can be alleviated by feeding fermented seaweed and seaweed plus terrestrial plants. To our knowledge, these are the first results showing how an energy supplement and plant based fermented products influences the redox balance, milk constituents, and fermentation parameters of ewes from late gestation through to weaning.

Table 6.5 Initial and final body weight and weight change of grazing ewes from late gestation to weaning that were either not fed concentrate (negative control; CON-) or fed concentrate (positive control; CON+), or CON+ with a seaweed extract (seaweed only; SWO), or CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP).

	Treatment [a]					Orthogonal Contrast *P*-value [b]		
Item	CON-	CON+	SWO	SWP	SE	SWO vs SWP	Eff. of Extract	Eff. of Supp.
Initial, kg	73.9	70.6	74.1	70.8	1.6	0.16	0.36	0.27
Weaning, kg	69.6	68.5	68.3	69.3	1.9	0.69	0.91	0.67
BW change, kg/d	-0.03	-0.01	-0.04	-0.01	0.01	0.04	0.35	0.50

[a] Treatments were not supplemented (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP)

[b] Orthogonal contrasts tested were SWO vs SWP = differences in the extracts used (SWO compared to SWP), Eff. Of Extract (CON+ compared to SWO and SWP), and Eff. of Supp. = effect of supplements (CON- compared to CON+, SWO, and SWP).

Table 6.2 Body weight and average daily gain of lambs from birth to weaning that were born to ewes either not supplemented (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP).

	Treatment [a]					Orthogonal Contrast *P*-value [b]		
Item	CON-	CON+	SWO	SWP	SE	SWO vs SWP	Eff. of Extract	Eff. of Supp.
Body Weight, kg								
Birth	4.8	4.9	5.0	4.8	0.1	0.14	0.98	0.61
Tailing	12.5	12.4	12.5	11.9	0.6	0.50	0.84	0.81
Weaning	31.3	30.3	29.9	30.5	1.0	0.63	0.91	0.38
ADG, kg per d								
Birth-to-Tailing	0.25	0.25	0.26	0.26	0.02	0.85	0.73	0.89
Tailing-to-Wean	0.28	0.28	0.27	0.28	0.01	0.28	0.91	0.56
Birth-to-Wean	0.27	0.27	0.26	0.28	0.01	0.25	0.94	0.68

[a] Treatments were not supplemented (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP)

[b] Orthogonal contrasts tested were SWO vs SWP = differences in the extracts used (SWO compared to SWP), Eff. Of Extract (CON+ compared to SWO and SWP), and Eff. of Supp. = effect of supplements (CON- compared to CON+, SWO, and SWP).

Table 6.3 Blood chemistry of ewes that either received no supplement (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP) from late gestation to weaning. Sampling dates were done prior to lambing (pre-lambing), after lambing (post-lambing), and at weaning.

	Treatment [a]					Orthogonal Contrast *P*-value [b]		
Item	CON-	CON+	SWO	SWP	SE	SWO vs SWP	Eff. of Extract	Eff. of Supp.
Urea, mmol/L								
Pre-lambing	8.07	8.14	7.11	7.89	0.50	0.25	0.26	0.50
4-wk in milk	7.13	6.85	7.04	8.07	0.57	0.17	0.27	0.76
Weaning	8.49	10.25	11.20	9.71	0.56	0.05	0.75	<0.01
TAS, mmol/L								
Pre-lambing	1.16	1.10	1.11	1.12	0.03	0.84	0.60	0.22
4-wk in milk	1.16	1.02	1.12	1.13	0.04	0.91	0.04	0.10
Weaning	1.14	1.11	1.16	1.14	0.04	0.81	0.45	0.89
GPx, U/mL								
Pre-lambing	15.25	11.97	13.98	13.49	2.9	0.91	0.62	0.53
4-wk in milk	14.85	39.35	34.09	29.22	4.0	0.36	0.05	<0.01

[a] Treatments were not supplemented (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP)

[b] Orthogonal contrasts tested were SWO vs SWP = differences in the extracts used (SWO compared to SWP), Eff. Of Extract (CON+ compared to SWO and SWP), and Eff. of Supp. = effect of supplements (CON- compared to CON+, SWO, and SWP).

Table 6.6 Rumen fermentation parameters of ewes sampled at weaning that either received no supplement (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP) from late gestation to weaning.

	Treatment [a]					Orthogonal Contrast *P*-value [b]		
Item, mmol/L	CON-	CON+	SWO	SWP	SE	SWO vs SWP	Eff. of Extract	Eff. of Supp.
NH_3	11.22	15.42	16.78	14.78	4.8	0.27	0.81	<0.01
Acetate	59.33	62.20	65.00	55.11	6.4	0.04	0.58	0.72
Propionate	14.51	16.46	15.85	14.52	1.9	0.36	0.28	0.37
Butyrate	7.07	8.30	9.38	7.61	0.9	0.02	0.75	0.04
Valerate	0.67	0.85	0.91	0.84	0.1	0.50	0.75	0.01
Hexanoate	0.06	0.03	0.06	0.07	0.01	0.77	0.04	0.74
Iso-Butyrate	0.86	0.99	1.09	1.03	0.16	0.48	0.28	0.02
Iso-Valerate	1.01	1.20	1.33	1.24	0.2	0.44	0.40	0.01
Acetate:Propionate	4.14	3.81	4.14	3.81	0.1	0.03	0.17	0.07
Total VFA	83.47	90.02	93.58	80.40	9.6	0.06	0.59	0.44

[a] Treatments were not supplemented (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP)

[b] Orthogonal contrasts tested were SWO vs SWP = differences in the extracts used (SWO compared to SWP), Eff. Of Extract (CON+ compared to SWO and SWP), and Eff. of Supp. = effect of supplements (CON- compared to CON+, SWO, and SWP).

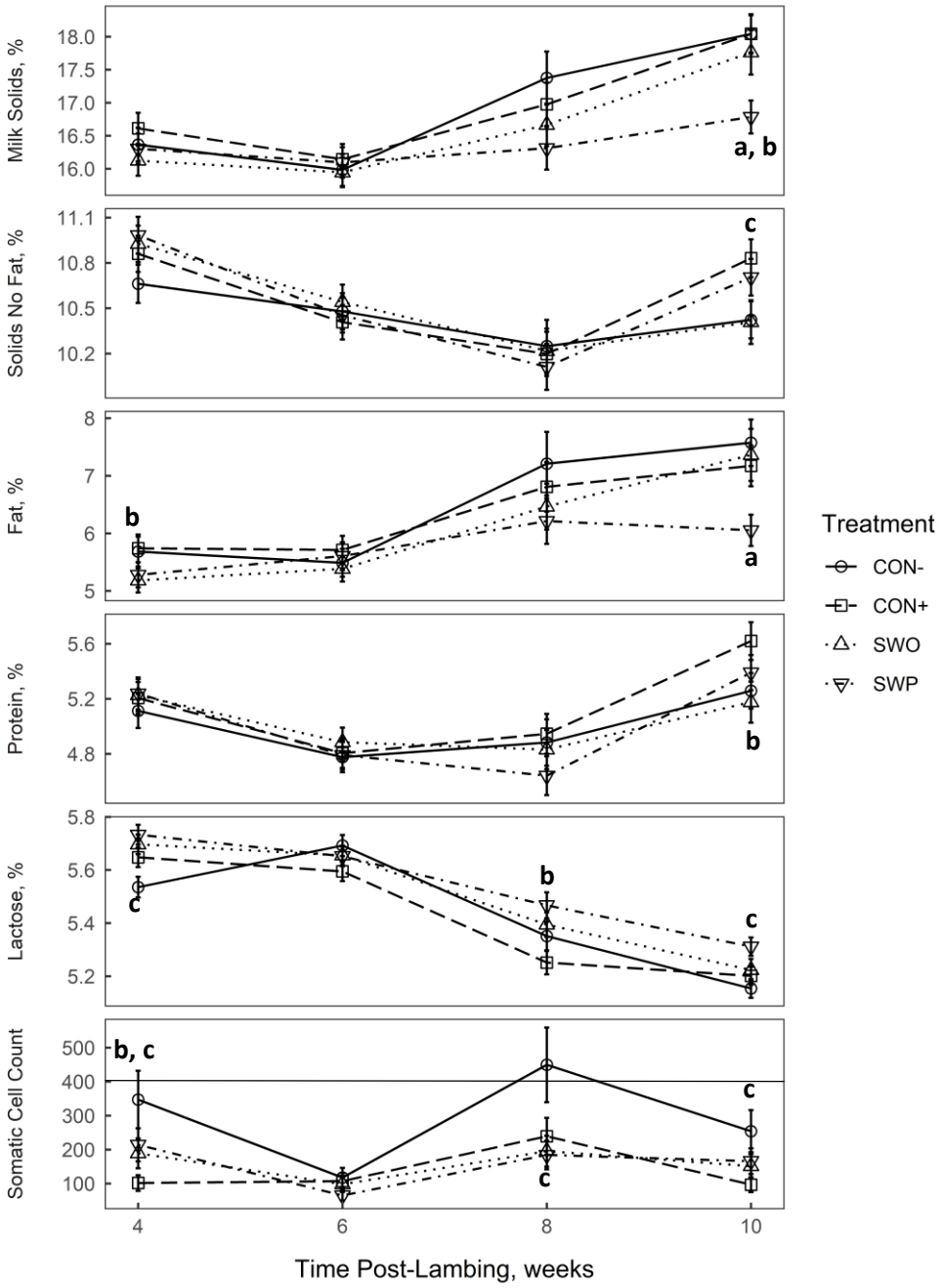

Figure 6.1 Milk composition 4-10 weeks of lactation from ewes that either received no supplement (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP) from late gestation to weaning. Superscripts within each panel within each week after lambing indicates significance ($P \leq 0.05$) for the orthogonal contrasts: [a]SWO vs SWP, [b]effect of extract (CON+ compared to SWO and SWP), and [c]effect of supplement (CON- compared to CON+, SWO, and SWP). Somatic cell count is in $1x10^3$/mL of milk and the flat line in the SCC panel is at the suggested threshold for subclinical mastitis ($400x10^3$ SCC/mL).

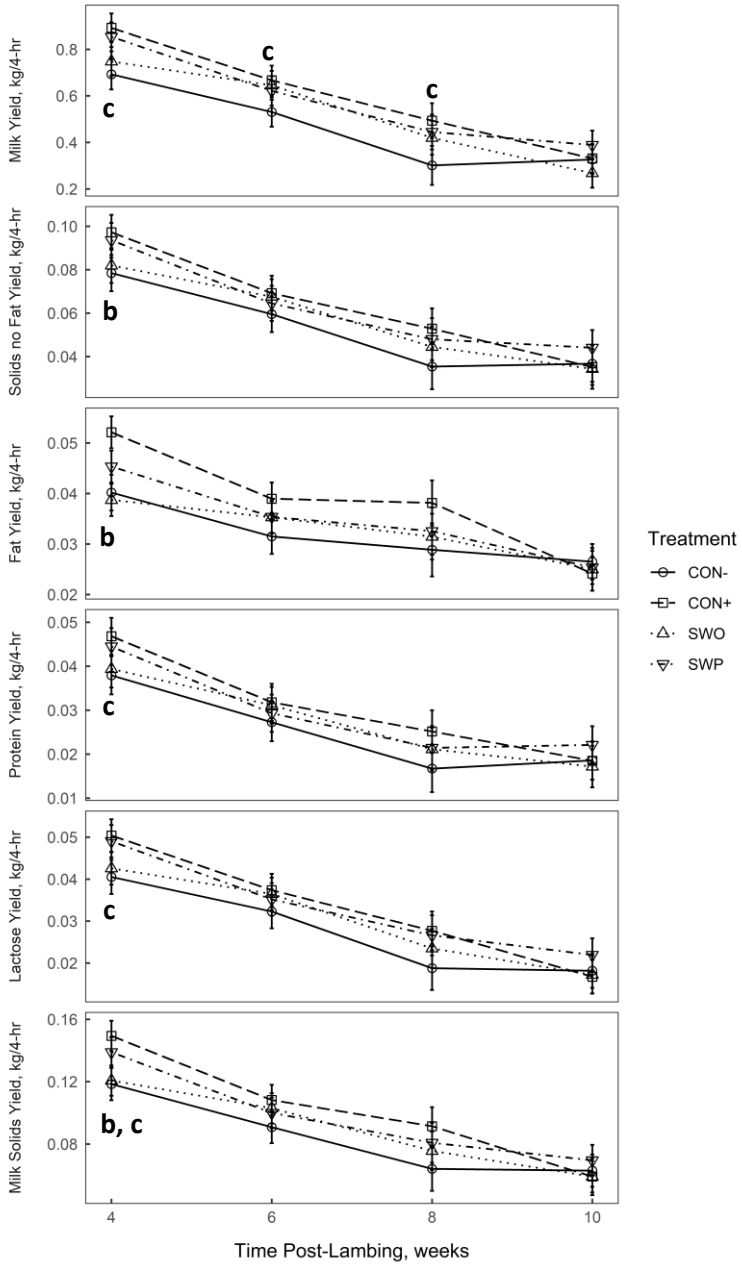

Figure 6.2 Milk yields 4-10 weeks of lactation from ewes that either received no supplement (negative control; CON-), a pelleted supplement only (positive control; CON+), CON+ with a seaweed extract (seaweed only; SWO), and CON+ with a seaweed plus terrestrial plant extract (seaweed plus; SWP) from late gestation to weaning. Superscripts within each panel within each week after lambing indicates significance ($P \leq 0.05$) for the orthogonal contrasts: [a]SWO vs SWP = differences in the extracts used (i.e. SWO compared to SWP), [b]effect of extract (i.e. CON+ compared to SWO and SWP), and [c]effect of supplement (i.e. CON- compared to CON+, SWO, and SWP).

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Chapter 7

Plant extracts provided to hogget ewes improves their lambs' antioxidant status at weaning.

7.1 Abstract

The objective of this experiment was to determine if supplementation with fermented plant extracts will affect the livestock performance (BW change and reproduction and antioxidant status of ewe lambs managed to lamb as yearlings (EWES). A further objective of this experiment was to determine how providing the fermented plant extracts may influence their lambs' (LAMBS) performance, rumen fermentation patterns, and antioxidant status following weaning. The EWES [n = 60; initial body weight (BW) = 29.6 ± 2.4] were provided a concentrate supplement (100 g/ewe/d) with either no extract (CON), a seaweed based extract (SWO; 10 mL/ewe/d), or a seaweed plus terrestrial plant extract (SWP; 10 mL/ewe/d), from their weaning through to the weaning of their lambs. There was a negative linear and a quadratic relationship and at d 360 there was a negative linear relationship between the number of lambs raised (NLR) and total antioxidant status (TAS; mmol equivalence/L) of the plasma. At weaning, there was a linear relationship between BW change and TAS, so that TAS = BW change (g/d) × 0.56 + 1.31 ($P < 0.01$; $R^2 = 0.36$). However, neither treatment nor NLR influenced glutathione peroxidase (GPx) activity ($P > 0.05$), indicating that while NLR and BW loss depleted TAS, the oxidative stress was effectively managed by all ewes. One day after weaning, the LAMBS born to SWO and SWP EWES had 13-14% greater ($P = 0.02$) TAS and had lower ($P = 0.03$) GPx activity than LAMBS born to CON EWES indicating greater antioxidant transmission to their offspring in SWO and SWP EWES. Overall, it was concluded that NLR and BW changes did not appear to be great enough to elicit oxidative stress in EWES. Supplying SWO and SWP to sheep increased the maternal transmission of antioxidants to their offspring.

Key words: Yearling ewe lambing; oxidative stress; stress at weaning

7.2 Introduction

In New Zealand, breeding of ewe lambs generally occurs at 7-9 months of age so that they lamb as 12-14 months old yearlings (Kenyon et al., 2014). This management strategy can have several benefits. Lambing from yearlings increases the ewe's life time productivity, increases the total number of lambs available for a farm to sell each year, reduces the maintenance energy cost associated with carrying the ewe to two years before their first offspring (Kenyon et al., 2014), and reduces environmental impacts by decreasing emission intensity (i.e. reduced greenhouse gas emissions per unit of product; Hegarty et al., 2010). Due to these benefits, the proportion of New Zealand ewe lambs mated in New Zealand increased from 13.6% in the 1990s to 30.5% in the early 2000s (Beef + Lamb New Zealand, 2013). Management decisions to mate ewe lambs include: the weight of the lamb and the availability of feed, but also some producers may not mate ewe lambs as they have lower reproductive performance, lower lamb survival rates, and even reduced future productivity if the ewes are not properly managed, among other management concerns (Kenyon et al., 2014). Improving the productivity of mating ewe lambs is the greatest management tool to reduce the environmental impacts of producing lamb (Hegarty et al., 2010), and to increase productivity of sheep (Kenyon et al., 2011).

Ewe lambs that are mated are required to budget nutrients towards personal growth, fetal development and growth, and then to raise the lambs produced. The nutrient demand of these fractions can influence their subsequent development, reproductive performance, flock longevity, and ultimately their and their lambs health (Morris et al., 2005). One health concern in scenarios where energy demand is high and nutrition is not being met properly is oxidative stress, thus linking diet and energy demand to health. For example, goats fed a higher energy dense diet (19.7% DM as corn) had greater plasma antioxidant capacity than goats fed a lower energy dense diet (5.6% DM as corn; Celi et al., 2010). In addition to the consideration of productivity and health of the yearling dam, there is concern around the health and subsequent post-weaning productivity of lambs born to yearling ewes. For instance Loureiro

et al. (2011) reported that lambs born to yearling ewes were 19.6% lighter at birth and 10.8% lighter at one year-of-age. Stressful events elicit oxidative stress (Beck and Gregorini, 2020) and, as such, weaning of lambs from yearling ewes, which may already have compromised growth and development, pose a significant health concern. Therefore, antioxidant support may be particularly needed for the offspring of yearling ewes.

Previous research on the effects of extracts obtained from fermented seaweed (*Ecklonia radiata*; SWO; Animal Health Tonic; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand) and seaweed plus terrestrial plants (SWP; Fortress+; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand) have demonstrated improved antioxidant status of periparturient dairy cows (Chapter 4). These supplements have also reduced the oxidative stress and improved antioxidant status of ewes fed grain based supplements at peak lactation (Chapter 6). Additionally, these products may alter ruminal volatile fatty acid (VFA) production (Chapter 4), reduce ruminal ammonia (NH_3) production (Beck et al., 2019), and urinary nitrogen (UN) excretion (Chapter 5). However, how these products may alter ruminal fermentation, N dynamics, or their effects on antioxidant status in breeding, gestating, or lactating yearling ewes has yet to be investigated. Further, the ability of these plant extracts to increase the transmission of maternal antioxidants to lambs born from yearling ewes or their subsequent rumen fermentation characteristics is also unknown. The objective of this experiment was to determine the effects of fermented seaweed extract (SWO) and fermented seaweed extract and terrestrial plant extracts (SWP) supplementation on ewe lambs managed to lamb as yearlings and their offspring at weaning, with regards to antioxidant status, rumen fermentation characteristics, and production (both body weight and reproductive). It was hypothesized that these products would improve the antioxidant status of yearling ewes and of their lambs and would improve animal performance by altering rumen fermentation. It was also hypothesized that the yearling ewes would experience some degree of oxidative stress and depletion of their antioxidant defense during lactation and that supplementation with fermented plant extracts would enhance their antioxidant defense.

7.3 Materials and Methods

This experiment was conducted at the Lincoln University Sheep Farm. All procedures involving animals described in this manuscript were approved by the Lincoln University Animal Ethics Committee (AEC 2018-25).

7.3.1 Animals and Treatments

This experiment was conducted over 360 days, from December 2018 through December 2019. The ewes in the current experiment were offspring of Coopworth ewes that were used in a previous experiment and data from these ewes from birth to weaning is reported previously (Chapter 6). During the last third of gestation, the dams of the ewes used in the current experiment were allocated to supplementation treatment groups and provided 100-g per head per d of a pelleted supplement (labeled to contain 12.2 MJ of metabolizable energy/kg DM, 12.2% CP, and 2% fat; Sheep Nuts, Reliance Feeds, Canterbury, New Zealand) with either no additions (CON), or 10-mL per ewe per d of a seaweed extract (SWO; Agrisea Ltd., Paeroa, New Zealand) or a seaweed plus terrestrial plant extract (SWP; Agrisea Ltd., Paeroa, New Zealand). The ewes were fed daily in their paddock replicates, which were composed of a predominately perennial ryegrass (*Lolium perenne* L.) and white-clover (*Trifolium repens* L.) based sward, until weaning.

Following weaning (14 December 2018), the ewe lambs [EWES; n = 60, 20 per treatment; initial body weight = 29.6 ± 2.4 (mean ± standard deviation)] were allocated to the current experiment based on body weight to ensure similar initial body weights. The EWES used in the current experiment were managed on the same treatment regime as their dams. The EWES were randomly allocated to replicated paddocks (0.38 ha; n = 12; 4 per treatment) with 5 ewes per paddock. The EWES remained in their paddock until their lambs (LAMBS) were weaned (9 December 2019). For mating, the treatments were grouped by treatment and 3 yearling rams were randomly assigned to each treatment (20 EWES per three rams). Mating began 125 days after weaning (18 April 2019) with the rams being removed and EWES placed back to their replicated paddocks 164 days after weaning (27 May 2019). The EWES were

shorn approximately 13 d prior to lambing and lambing occurred from 18 September to 21 October 2019. The LAMBS were tailed (removal of tail by hot-iron) 326 d after the EWES were weaned. The LAMBS were weaned at 360 d after the EWES were weaned. Finally, LAMBS were vaccinated against *Clostridium perfringens* at tailing and again at weaning by subcutaneously injecting 2-mL of vaccine (Lamb Vaccine, Coopers, Upper Hutt, New Zealand).

7.3.2 Measurements and Sample Collection

Forage sampling was conducted on d 56, 178, 279, 328, and 360 after EWES were weaned. Forage mass was estimated using a rising plate meter (Jenquip, Feilding, New Zealand) and the calibration equation developed from these same paddocks is described in Chapter 6. Additionally, hand grab samples of herbage were cut to ground level in 10 random locations in each paddock during each forage measurement day. A subsample of the herbage sample was oven dried at 60°C to determine DM percentage. The remainder of the herbage samples were frozen (-20°C), freeze-dried, and then ground to pass through a 1-mm screen using a centrifugal mill (ZM200 Retsch).

Yearling ewe body weight was measured at 0, 53, 125, 164, 280, 326, and 360 d after EWES weaning for all animals. During lambing, LAMBS were weighed once in the morning (0800) of their birth date, sexed, and allocated a unique electronic identification tag. All LAMBS were then weighed at tailing (d 326) and finally at weaning (d 360).

Animal samples from EWES were collected from three randomly selected focal animals per paddock (total of 36). Blood samples were collected on d 0, 125, 280, 326, and 360 after EWES were weaned. Additionally, three LAMBS per paddock were randomly selected and sampled at weaning. Blood samples were collected into a 10-mL lithium heparinized blood tube via jugular venipuncture. A subsample of heparinized whole blood was removed and stored at -20 ºC. The blood was then centrifuged at 3000 x g for 15-min at 4 ºC and plasma was aspirated and stored at -20 ºC. Ruminal fluid was collected from EWES on 0, 125, and 360 d after EWES were weaned and ruminal fluid from LAMBS at d 360 by esophageal tubing. Two mL of ruminal fluid was transferred into a 2-mL tubes without

sulfuric acid [for determination of VFA concentration] and another subsample of ruminal fluid was transferred into a 2-mL Eppendorf tube, which was acidified with approximately 30 μL sulfuric acid (95% v/v; Fisher Scientific; Loughborough, United Kingdom) to prevent NH_3 volatilization.

7.3.3 Sample Analysis

Forage samples were analyzed for nutritive quality by near infrared spectroscopy (NIRS; FOSS NIR Systems 5000, Maryland, USA). Nutritive values used to develop the calibration equations ($R^2 > 0.90$) were obtained for DM (AOAC, 1990; method 930.15) and ash (AOAC, 1990; method 942.05), NDF (Van Soest et al., 1991), ADF (method 973.18; AOAC, 1990), CP by combustion (Variomax CN Analyser, Elementar), water soluble carbohydrates (MAFF, 1986), and DM and OM digestibility (Iowerth et al., 1975).

Glutathione peroxidase (GPx) activity in the heparinized whole blood was determined according to a commercial kit manual (Randox, Nishinomiya, Japan; RANSEL, catalog number RS5074). Total antioxidant status (TAS) of plasma was determined using a colorimetric method using a commercially available kit (Randox, Nishinomiya, Japan; catalog number NX2332). Plasma urea was determined using an enzymatic kinetic technique following the kit manual (Randox, Nishinomiya, Japan; catalog number UR9781). Ruminal NH_3 concentration was determined by an enzymatic UV method using a commercially available kit (Randox, Nishinomiya, Japan; catalog number AM3979). All of the Randox kits were analyzed using an automated clinical analyzer (Daytona RX; Randox, Nishinomiya, Japan). Finally, ruminal VFA concentrations were determined using a gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) fit with a SGE BP21 30 m x 530 μm x 1.0 μm bore capillary column (Chen and Lifschitz, 1989).

7.3.4 Statistical Analysis

Prior to analysis all variables were checked for normality and homogeneity of variances using a Shapiro-Wilk and Bartlett's test, respectively (R Core Team, 2020, v.3.6.3). Forage allowance and nutritive quality of the forages were analyzed using a fixed-effects generalized linear model (GLM) with repeated measures, with a gamma distribution. Forage mass was analyzed using a fixed-model analysis

of variance (ANOVA) with repeated measures. Both the ANOVA and GLM models included treatment, day, and treatment by day interaction as fixed effects. The least-squares means were determined and back-transformations were done (for the GLM models only) using an estimated marginal means (emmeans) package (Lenth, 2018).

The EWES body weight and average daily gain were analyzed by a mixed model ANOVA linear effects model using the 'lme4' package (Bates et al., 2015). These models included treatment, day, the number of lambs the ewes were raising, and all possible 2 and 3-way interactions as fixed effects and the individual animal as random effects. After a significant ANOVA, contrasts were generated comparing the treatments within the number of lambs raised (i.e. none, single, or twins; NLR) within measurement day and polynomial contrasts (linear and quadratic) were generated to test the NLR within treatments with day. The NLR was included as a factor so that any trends in the data prior to mating would suggest a relationship influencing reproductive success and any relationship after mating could be inferred as being a result of the effect of NLR. The LAMBS body weight at birth, tailing, and weaning and weight change (from birth to tailing, tailing to weaning, and birth to weaning) were analyzed using mixed model ANOVA, with treatment, sibling status (either twin or single), sex, and all possible two-way and the three-way interaction as fixed effects, with their dam as a random effect.

All reproductive performance data was calculated for each paddock within treatment (n = 4 paddocks per treatment). These values were done for lambing date past their weaning date, pregnancy rate (proportion of pregnant ewes/5), number of lambs gestated per pregnant ewe, number of lambs born live per pregnant ewe, number of lambs weaned per pregnant ewe, and number of lambs died per pregnant ewe. Data were analyzed using a GLM with a gamma distribution and treatment as the only fixed effect. These data were back-transformed and means were determined using an emmeans package.

Both plasma urea, TAS, and GPx of EWES were analyzed using a generalized linear mixed effects model (GLMER) function and a gamma distribution that included treatment, sampling day, NLR, and all

possible two and three-way interactions as fixed effects and individual animal as random effects. All rumen fermentation data of EWES were analyzed using mixed models with treatment, sampling day, and their interaction as fixed effects and individual animal as random effects. Total VFA concentration was analyzed using a mixed model ANOVA with the 'lmer' function. Acetate to propionate ratio and each individual VFA as a percentage of the total was analyzed using the 'glmer' function with a gamma distribution. Least-squares means were determined and for the 'glmer' models means were back-transformed using the 'emmeans' function of R.

The components analyzed within LAMBS blood, and ruminal fluid were averaged within paddock and found to be normally distributed ($P > 0.10$) and to have homogenous variances between treatments ($P > 0.10$). The samples from LAMBS were analyzed using a fixed effects ANOVA, with treatment as the only fixed effect. For all data statistical analysis was conducted in R (R Core Team, 2020, v.3.6.3) and statistical significance was declared at $P \leq 0.05$ and tendencies are discussed at $0.05 < P \leq 0.10$.

7.4 Results

7.4.1 Forage Measurements

The SWO treatment had a lower ($P < 0.01$) forage mass compared with the other treatments (Table 7.1). Despite the lower herbage mass, forage allowance (kg of forage DM per kg of BW) was not influenced ($P = 0.78$) by the treatments. There was no significant day × treatment interactions ($P \geq 0.66$), nor was the main effect of treatment significant ($P \geq 0.21$) for any of the forage nutritive composition variables measured (Table 7.1).

7.4.2 Ewe Body Weight and Body Weight Change

For BW and BW change, there was no three-way interaction between supplement treatment, measurement day, and NLR ($P \geq 0.46$; Table 7.2). However, there were significant day × NLR ($P < 0.01$) and day × supplement treatment interactions ($P \leq 0.05$). Accordingly, supplement treatment differences will be discussed within NLR within day and effects of NLR will be discussed within treatment within day.

7.4.2.1 Treatment differences

All supplement treatment groups had BW which were not different ($P > 0.10$), from EWES weaning up until 280 d after weaning (approximately 2 weeks prior) to lambing (Table 7.2). At day 280, SWP EWES weighed more ($P < 0.05$) than CON and SWO EWES, with the later not differing ($P > 0.10$). On days 326 and 360, CON and SWP EWES did not differ ($P > 0.10$) in BW, and were both greater ($P < 0.05$) than SWO EWES.

Between days 125 to 164, SWP EWES had greater ($P < 0.05$) BW change than SWO EWES, whereas CON EWES was intermediate and not different ($P > 0.10$) from the other two supplement treatments. From days 326 to 360, SWP and CON EWES did not differ ($P > 0.10$) in BW change, but they had greater ($P < 0.05$) BW change than SWO EWES.

7.4.2.2 Yearling ewe differences between the numbers of lambs raised

From weaning of these yearling ewes to the end of their mating, NLR did not ($P > 0.10$) influence BW nor BW change across all treatments (Table 7.2). At day 280 (prior to lambing), there were linear and quadratic increases in BW across NLR. During days 326 and 360, there were linear decreases in BW across NLR. From the end of mating to shearing prior to lambing, there were linear increases in BW change across NLR. From days 280 to 326 (after lambing), NLR resulted in linear reductions in BW change. Finally, from days 326 to 360 (weaning), there were linear and quadratic reductions in BW change as NLR increased.

7.4.3 Reproductive Performance

Lambing occurred approximately 292 days after the EWES were weaned and this was not influenced ($P = 0.17$) by supplement treatment (Table 7.4). While there was a tendency for SWP to have a greater ($P = 0.10$) pregnancy rate compared with SWO, there were no other treatment differences detected for reproductive performance.

7.4.4 Ewe Blood parameters

For GPx, there was an effect of NLR × sampling day interaction ($P < 0.01$). There was a quadratic effect ($P < 0.01$) on GPx activity for NLR on the sampling day measured prior to lambing, where the GPx activity was 29.9, 17.6, and 38.5 for non-pregnant, single, and twin bearing EWES, respectively. For all other time points there were no linear ($P \geq 0.23$) or quadratic ($P \geq 0.48$) effects of NLR for GPx activity.

Fifty-three and 125 days after the EWES were weaned, SWP had a lower ($P < 0.05$) TAS than SWO, while CON was numerically intermediate and not different ($P > 0.10$) than the other two treatments (Table 7.5). There were no other supplement treatment differences ($P > 0.10$) detected for TAS throughout the remainder of the experiment. The NLR did not ($P > 0.10$) influence TAS until after lambing. Approximately four weeks after lambing (d 326), NLR had a linear and quadratic relationship ($P < 0.05$) with TAS, so that EWES with no lambs had, on average, 15.7% and 11.4% greater TAS than single and twin bearing EWES, respectively. At weaning there was a negative relationship between NLR and TAS for all treatments, where no-lamb bearing EWES had, on average, 10.5% and 13.4% greater TAS than single and twin bearing ewes, respectively.

Plasma TAS, measured at weaning (d 360), was determined to be positively related ($P < 0.01$) to the EWES BW change from d 326 (i.e. tailing) to d 360 (Figure 7.1). For every 1 kg of BW gain, TAS increased by 0.56 mmol equivalence per L ($R^2 = 0.36$). At the intercept, i.e. a 0 kg/d BW change, TAS was determined to be 1.31 mmol/L. This relationship was consistent across treatments and NLR, as neither significantly interacted with the regression slope or intercept.

Differences between plasma urea and the supplementation treatments was largely inconsistent across NLR and sampling days (Table 7.5). Fifty-three days after EWES were weaned, the SWP ewes that did not later become pregnant had a greater ($P < 0.05$) plasma urea concentration than CON and SWP, and CON and SWP were not different ($P > 0.10$). For the EWES that would bear a single lamb, SWO had a greater ($P < 0.05$) plasma urea concentration than SWP, and CON was numerically intermediate and not different ($P > 0.10$) from SWP or SWO. The EWES that would bear twins were not different ($P > 0.10$)

between treatments 53 days after weaning. For the sampling taken 125 days after weaning, the only treatment difference determined was for EWES, which would end up not becoming pregnant, where SWP had a greater ($P < 0.05$) plasma urea concentration compared with SWO and CON, which were not different ($P > 0.10$) from each other. At tailing (d 326), there were no treatment effects ($P > 0.10$) for EWES with single or no lambs; however, for the EWES with twins, SWO had a greater ($P < 0.05$) plasma urea concentration than SWP, while CON was intermediate and not different ($P > 0.10$) from the other two treatments. Finally, at weaning, the only treatment difference for plasma urea concentration was observed for EWES with no lambs, where SWP was greater ($P < 0.05$) than CON, and SWO was intermediate and not different ($P > 0.10$) from the other treatments.

The CON EWES did not exhibit a linear or quadratic relationship ($P > 0.10$) between NLR and plasma urea concentration. For the SWO treatment, there was a negative linear relationship ($P < 0.05$) between NLR and plasma urea only at weaning. The SWP treatment had a linear relationship ($P < 0.05$) between NLR and plasma urea for all sampling days, with the exception ($P > 0.10$) of sampling day 280. Finally, in addition to the significant linear relationship, SWP EWES had a quadratic relationship ($P < 0.05$) between NLR and plasma urea on sampling day 53.

7.4.5 Ewe and Rumen Fermentation Patterns

There was no main effect of NLR and any interactions which included NLR ($P > 0.10$) for any of the rumen fermentation data and was removed from the analysis. When EWES were weaned, SWO had a greater concentration of ruminal NH_3 than SWP, while CON was intermediate and not different from the other treatments (Table 7.6). There was an interaction ($P = 0.05$) between treatment and sampling day. At d 53, CON had a greater ($P < 0.05$) total volatile fatty acid (VFA) concentration than SWP, while SWO was intermediate and not different ($P > 0.10$) from SWP and CON. There were no treatment differences at d 125. At d 360, SWO had a greater ($P < 0.05$) total VFA concentration than CON, whereas SWP was intermediate and not different ($P > 0.10$) from the other treatments. There was a sampling day by treatment interaction ($P = 0.04$) for the acetate to propionate ratio. At d 53 and 125,

SWO had a greater ($P < 0.05$) acetate to propionate ratio than CON, while SWP was not different ($P > 0.10$) from either CON or SWO. For the major VFA (i.e. acetate, propionate, and butyrate) there were no observed main effects of treatment ($P \geq 0.13$) or a sampling day by treatment interaction ($P \geq 0.06$). There were treatment differences observed for the minor VFA; however, there was a lack of consistency seen in these effects. For example, CON had a greater ($P < 0.05$) proportion of their VFA as valerate compared to both SWO and SWP, while SWO and SWP were not different ($P > 0.10$). During sampling d 125 the proportion of VFA as valerate for CON and SWP were not different ($P > 0.10$) and were both greater ($P < 0.05$) than SWO. On day 360, SWO had a greater ($P < 0.05$) valerate proportion than CON, while SWP was numerically intermediate and not different ($P > 0.10$) form CON or SWO. On d 53 and 125, CON had greater ($P < 0.05$) hexanoate proportion of total VFA than SWP, and SWO was not different ($P > 0.10$) from CON or SWP. There were no ($P > 0.10$) treatment differences for hexanoate at d 360. There was a tendency ($P = 0.08$) for SWP to have greater proportions of iso-butyrate than CON on d 125, and for SWO and CON on d 360. The SWP EWES did have a greater ($P < 0.05$) proportion of iso-valerate than SWO, and CON was not different ($P > 0.10$) from either treatment on d 125. The SWP ewes had greater ($P < 0.05$) proportions of iso-valerate than CON and SWO on d 360.

7.4.6 Lamb Body Weight and Weight Change

There were no treatment differences ($P = 0.41$) for LAMBS birth weight. The LAMBS born to SWO ewes tended ($P = 0.10$) to have greater BW at tailing and weaning, but this trend disappeared when lambing date was included in the model as a covariate, so that there were no treatment differences ($P = 0.92$) detected for BW at any of the measurement times. There were additionally no treatment differences ($P \geq 0.26$) for average daily gain at any time point.

There was a tendency for rams to have a greater ($P < 0.10$) birth and weaning BW than ewe LAMBS (Table 7.7). Ram LAMBS tended ($P = 0.09$) to have a greater BW gain from birth to tailing, did have a greater ($P = 0.01$) BW gain from tailing to weaning, and tended ($P = 0.07$) to have greater weight

gain from their birth to weaning compared with the ewe LAMBS. Single LAMBS had a greater ($P < 0.01$) BW at all measurement times compared with twin LAMBS. Single LAMBS additionally had greater ($P < 0.01$) average daily gain than twin LAMBS.

7.4.7 Lamb Blood Parameters

The LAMBS born to EWES provided SWO and SWP had 29.0% and 32.0% less ($P = 0.03$) blood GPx activity compared with LAMBS born to CON EWES, respectively (Table 7.8). Accordingly, SWO and SWP LAMBS had 13.0% and 14.8% greater ($P = 0.02$) plasma TAS than CON LAMBS, respectively. Finally, SWO and SWP LAMBS had 14.8% and 15.6% greater ($P = 0.04$) plasma urea concentration than CON LAMBS. There existed a negative linear ($P = 0.03$; $R^2 = 0.40$) relationship between plasma TAS and blood GPx activity, where plasma TAS (mmol equivalence/L) = GPx (U/L of whole blood) × -0.01 + 1.52 (Figure 7.2).

7.4.8 Lamb Rumen Fermentation Characteristics

The SWP LAMBS had a greater ($P < 0.01$) total VFA concentration and a lower acetate to propionate ratio of rumen fluid than both CON and SWO, while CON and SWO did not ($P > 0.10$) differ (Table 7.8). The SWP LAMBS had a lower acetate proportion of total VFA than SWO, whereas CON was intermediate and did not differ ($P > 0.10$) from either treatment. The SWP LAMBS had a greater ($P < 0.05$) propionate and valerate, and lower branch-chain VFA (i.e. iso-butyrate and iso-valerate) proportions than CON and SWO, with the later not differing ($P > 0.10$). There were no differences ($P = 0.47$) in the butyrate proportion between treatments and SWO and SWP tended ($P = 0.10$) to have greater proportions of hexanoate than the CON LAMBS.

7.5 Discussion

It was hypothesized that SWO and SWP would improve the antioxidant status of EWES, following lambing and would improve animal performance by altering rumen fermentation. This hypothesis was not accepted, as there were no supplement treatment effects on reproductive performance, nor consistent effects of supplement on rumen fermentation and BW throughout the experiment. It was also hypothesized that EWES would have lower total antioxidant status as NLR increased and that this would

be related to BW changes. This hypothesis is accepted. However, the hypothesis that EWES would experience oxidative stress based on the relative high total antioxidant status of all of the EWES and due to the lack of effect of NLR on glutathione peroxidase activity is rejected. It was hypothesized that the SWO and SWP treatments would increase the LAMBS growth and performance, and this hypothesis is also rejected. Finally, it was hypothesized that LAMBS born to EWES provided SWO and SWP would experience less oxidative stress compared with the CON LAMBS. This hypothesis is accepted due to the LAMBS in the SWO and SWP treatment groups having greater plasma TAS and lower blood GPx activity.

7.5.1 Ewe Reproductive Performance and Ewe and Lamb Weight Change

The reproductive performance in the current experiment is similar to other experiments focused on yearling ewes. In the current study, pregnancy rates did not differ between treatments and ranged from 65% to 90%, which are similar to the ranges reported previously (Kenyon et al., 2010). There were 1.05 to 1.45 lambs gestated per pregnant ewe in the current study, which is similar to the 1.13 to 1.40 lambs gestated per pregnant ewe reported by Muñoz et al. (2009). Additionally, there were 0.80 to 1.15 lambs weaned per pregnant ewe in the current study, which is also similar to rates reported for yearling ewes in previous literature (0.76 to 1.08; Muñoz et al., 2009). Yearling ewes are known to have greater difficulty caring for their offspring compared to mature ewes (Corner et al., 2013). Yearling ewes have lambs with much higher mortality rates, which is dependent on if the lamb is a single or twin, and, if a twin lamb, how that lambs BW compares to its sibling (Schreurs et al., 2010). It was determined that a single lamb has a 22.1% mortality, heavy twins (heavier than its sibling) had a mortality rate of 22.4%, and lighter twins (lighter than their siblings) had a 28.5% mortality rate (Shreurs et al., 2010). These are similar to the mortality rates (Number of lambs lost per pregnant ewe divided by number of lambs gestated per pregnant ewe) observed in the current study, which ranged between 11.5% and 23.8%.

Differences in BW by NLR was only detected following the weigh day, approximately 2 weeks prior to lambing. Ewe BW increased linearly for all treatments or quadratically (CON ewes only) across NLR when they were weighed on d 280 (Table 7.2). Prior to lambing, pregnant ewes have additional

weight due to fetal, placental, and mammary tissue development (Gootwine et al., 2007). Accordingly, the greater BW of ewes as NLR increased is likely due to the additional weight resulting from gestation. Approximately 4 weeks after lambing and at weaning there were linear reductions in BW as the ewes raised more lambs (none to single to twins). Accordingly, there were linear reductions in weight change from prior to lambing to tailing and from tailing to weaning as the number of lambs raised increased. The linear losses in weight change from before lambing to 4 weeks after lambing was likely driven by losses in fetal and placental weight, plus differences in mobilization of body stores for milk production. Whereas the differences in weight change from tailing to weaning is due to differences in energy demand for milk production. Obviously, the ewes which were not raising lambs had no energy demand for milk production and as such they gained 133 and 169 g/d more than ewes bearing singles and twins, respectively. Further, milk production has been shown to increase by as much as 17% for Rambouillet, Columbia, and Polypay breeds and 61% for the Suffolk breed, for ewes bearing twins compared with singles (Snowder and Glimp, 1991). This is due to the added demand for milk by their offspring. As such, it has been said that single lambs represent *ad libitum* milk intake by lambs, whereas milk intake by twins represent the maximum milk production by the ewe (Morgan et al., 2007). Thus, this additional energy demand for milk production by twin bearing ewes likely accounts for the difference in ewe BW at weaning and weight change from tailing to weaning compared with single bearing ewes.

7.5.2 Ewe antioxidants

As mentioned above, as NLR increases, the energy demand on the ewe increases, largely due to increased milk production. This resulted in quadratic and linear reductions in TAS as NLR increased 4 weeks after lambing and linear reductions at weaning, independently of treatment. Increased energy demand associated with NLR was likewise seen in differences in BW change after lambing, with linear reduction in weight change after lambing and at weaning with increased NLR. As NLR increased there were reductions in TAS and BW change. Accordingly, there was a positive linear relationship determined between TAS and BW change at weaning for EWES, thus supporting that antioxidant depletion is related

for metabolic demands (Figure 7.1). Increased milk production has been associated with increased production of oxidants, due to the greater metabolic demand and mobilization of energy stores (Castillo et al., 2003; Gabai et al., 2004; Lohrke et al., 2004). These oxidants then deplete the host's antioxidant defenses. For instance, dairy cows that gave birth in the summer experienced more heat stress, which induced greater lipid peroxidation by products and higher antioxidant enzyme activity, indicating oxidative stress, and depleted glutathione (a non-enzymatic antioxidant) levels compared with cows who calve in the spring (Bernabucci et al., 2002). This can be seen in the current study by the positive relationship between plasma TAS and BW change. Thus, the results from the current experiment confirm that the increased energy demand associated with increasing NLR depletes the antioxidant defenses of yearling ewes after lambing.

Although the EWES had depleted TAS according to NLR and BW change in the current study, they were apparently able to effectively manage the oxidants induced by the metabolic demands, so that oxidative stress may not have occurred. This can be seen by the relatively high plasma TAS seen for all EWES compared to previous experiments. For example, the EWES' dams (all twin bearing mature ewes) had TAS values of 1.02 to 1.16 compared with the values measured in the current study after lambing, 1.28 to 1.52 (Chapter 6). The lack of differences observed in GPx activity in the current experiment further highlight the ability of the EWES to manage the oxidative stress experienced. Antioxidant enzymes, e.g. GPx, are upregulated by several oxidants, thus their activity has been suggested as a marker of oxidative stress (Celi, 2011). While EWES were able to effectively manage the oxidative stress in the current experiment, so that GPx activity was not increased, some of the ewes' antioxidants were depleted due to metabolic demands, which can be seen by the linear relationship between TAS and BW change. However, we suggest that the depletion of antioxidants seen in the current experiment was not great enough to induce oxidative stress, which explains the lack of benefit provided by the extracts. This may explain why the plant extracts (SWO and SWP) did not show antioxidant benefits in the current

study, where they have in previous research with periparturient dairy cows (Chapter 4) and with sheep during peak lactation (Chapter 6).

7.5.3 Lamb antioxidants at weaning

Contrary to EWES, one day after weaning, LAMBS born to CON EWES had a lower TAS and greater GPx activity than LAMBS born to SWO and SWP EWES. Further, there was a negative linear relationship between TAS and GPx activity (Figure 7.2), which supports that these LAMBS were experiencing some degree of oxidative stress. Weaning is known to be a physiologically stressful event (Rhind et al., 1998) and physiological stress can lead to the production of oxidants, which depletes antioxidant defense mechanisms (Beck and Gregorini, 2020). For instance, oxidative stress has been shown to increase due to common animal handling practices which are known to cause physiological stress in livestock (Chirase et al., 2004; Fidan et al., 2009; Fidan et al., 2010). This would indicate that supplementing ewes with these plant extracts imparts antioxidant defense to their offspring that may have important health implication to lambs, thereby reducing morbidity and mortality.

The transmission of antioxidants from dams to their offspring has been shown in several experiments. When ewes are fed a rosemary (*Rosmarinus officinalis* L.) or thyme (*Thymus zygis* ssp. *gracilis*) by-product across gestation and until weaning, their lambs have meat with greater antioxidant status compared with control lambs (Nieto et al., 2010a; Nieto et al., 2010b; Nieto et al., 2011). As with the current study, the lambs were not allowed to consume the dietary treatments directly, thus the antioxidant benefits must come from maternal transmission. This transmission may occur during gestation, through ingestion of antioxidants in their mother's milk, or both. Antioxidant minerals (Abdelrahman and Kincaid, 1995) and vitamins (Capper et al., 2005) have been shown to be transferred across the placenta. Additionally, the intake of antioxidant polyphenols has been related to polyphenol concentration (Besle et al., 2010) and subsequently antioxidant status of milk (Di Trana et al., 2015), thereby providing another means of maternal transmission of antioxidants. Maternal transmission of antioxidants appears to be an important aspect of ruminant health, but also in meat and milk quality.

Ultimately, the current study indicates that SWO and SWP, when fed to dams improves the antioxidant transmission to their offspring, which may provide health benefits following physiologically stressful events.

7.6 Conclusions

To our knowledge this is the first experiment to report linear reduction in antioxidant status by yearling ewes based on number of lambs raised and BW change after lambing. Additionally, this study, to our knowledge, is the first to show that providing yearling ewes with plant extracts, i.e. SWO and SWP, improves the antioxidant status of their lambs after weaning. As the lambs were precluded from the dietary treatments, the greater TAS and lower GPx activity of lambs born to ewes provided SWO and SWP, compared with CON lambs, indicates that these plant extract products increased the maternal transmission of antioxidants. These results could have important health implications, which may reduce the morbidity and mortality of lambs, thereby improving the overall productivity of the lamb industry.

Table 7.1 Forage mass, allowance, and nutritive composition for hogget ewes provided grain based supplements (100 g/head/d) with either no extract (CON) or 10 mL per head per d of a seaweed (SWO) or a seaweed plus terrestrial plant (SWP) extracts. The data presented are least-squares means from forage measurements (n = 5) throughout the experiment, from their weaning to weaning their lambs. Forage measurements were done in conjunction with other sampling days.

	Treatments				*P*-value		
Item [a]	CON	SWO	SWP	SEM	Treat.	Day	Inter
Forage Mass, kg DM/ha	2497.9[a]	2,230.3[b]	2,517.9[a]	64.7	<0.01	<0.01	0.58
Forage Allowance	2.91	2.83	2.89	0.08	0.78	<0.01	0.88
Nutritive value, %							
DM, % as-is	19.3	21.8	20.7	1.0	0.21	<0.01	0.66
OM, % DM	92.2	92.4	92.0	0.22	0.34	<0.01	0.97
CP, % DM	13.9	13.9	14.4	0.49	0.67	<0.01	0.86
NDF, % DM	44.8	44.5	43.5	0.84	0.54	<0.01	0.89
ADF, % DM	25.3	25.2	24.6	0.42	0.52	<0.01	0.74
WSC, % DM	20.6	20.8	20.6	0.44	0.90	<0.01	0.85
DMD, % DM	75.2	75.6	76.5	0.68	0.41	<0.01	0.82
OMD, % DM	79.9	80.3	81.3	0.79	0.44	<0.01	0.79

[a] DM = dry matter; Forage allowance is in kg DM/kg of body weight; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; WSC = water soluble carbohydrates; DMD = dry matter digestibility; OMD = organic matter digestibility.

Table 7.2 Body weight (kg) of hogget ewes provided grain based supplements (100 g/head/d) with either no extract (CON) or 10 mL per head per d of a seaweed (SWO) or a seaweed plus terrestrial plant (SWP) extracts.

	Treatments				ANOVA	
Item [a]	CON	SWO	SWP	SEM	Term	*P*-value
Day 0 (their weaning)					TRT	0.04
NONE	30.2	29.3	29.9	1.57	No. lambs	0.17
SINGLE	29.4	28.5	29.3	1.42	Day	<0.01
TWIN	30.1	29.2	30.0	1.38	TRT x No. Lambs	0.66
Day 53					TRT x Day	<0.01
NONE	39.0	37.1	38.7	1.56	No. Lambs x Day	<0.01
SINGLE	39.6	37.7	39.3	1.41	3-way interaction	0.46
TWIN	40.1	38.3	39.9	1.38		
Day 125 (begin mating)						
NONE	43.9	44.5	43.8	1.56		
SINGLE	45.8	46.4	45.8	1.41		
TWIN	45.8	46.4	45.7	1.38		
Day 164 (end of mating)						
NONE	46.6	45.7	47.7	1.40		
SINGLE	47.7	46.8	48.8	1.26		
TWIN	47.8	46.9	48.8	1.24		
Day 280 (prior to lambing)	*,**	*,**	*,**			
NONE	54.0[b]	51.9[b]	56.8[a]	1.56		
SINGLE	60.2[b]	58.1[b]	63.0[a]	1.41		
TWIN	60.6[b]	58.6[b]	63.5[a]	1.38		
Day 326 (after lambing)	*	*	*			
NONE	69.1[a]	65.2[b]	70.7[a]	1.56		
SINGLE	65.4[a]	61.4[b]	67.0[a]	1.41		
TWIN	57.7[a]	53.8[b]	59.4[a]	1.38		
Day 360 (weaning)	*	*	*			
NONE	75.6[a]	69.9[b]	76.9[a]	1.56		
SINGLE	67.3[a]	61.6[b]	68.7[a]	1.41		
TWIN	58.4[a]	52.7[b]	59.8[a]	1.38		

[a-c] means within a row without similar superscripts differ ($P < 0.05$).

[*] indicates a linear relationship, [**] indicates a quadratic relationship between number of lambs raised and body weight, within treatment within day.

Table 7.3 Body weight change (kg/d) for hogget ewes provided grain based supplements (100 g/head/d) with either no extract (CON) or 10 mL per head per d of a seaweed (SWO) or a seaweed plus terrestrial plant (SWP) extracts.

	Treatments				ANOVA[1]	
Item	CON	SWO	SWP	SEM[2]	Term	*P*-value
Day 0 to 53					TRT	0.03
NONE	162.9	146.8	159.9	23.1	No. lambs	<0.01
SINGLE	188.6	172.5	185.5	20.9	Period	<0.01
TWIN	186.3	170.2	183.2	20.4	TRT x No. Lambs	0.85
Day 53 to 125					TRT x Period	0.05
NONE	67.5	101.7	70.9	23.0	No. Lambs x Period	<0.01
SINGLE	86.8	121.0	90.2	20.8	3-way interaction	0.77
TWIN	78.17	112.3	81.5	20.4		
Day 125 to 164						
NONE	70.5^{ab}	31.5^{b}	98.5^{a}	23.0		
SINGLE	48.9^{ab}	9.9^{b}	76.9^{a}	20.8		
TWIN	51.9^{ab}	12.9^{b}	79.9^{a}	20.4		
Day 164 to 280	*	*	*			
NONE	63.3	53.4	78.7	23.0		
SINGLE	107.4	97.5	122.8	20.8		
TWIN	110.7	100.8	126.1	20.4		
Day 280 to 326	*	*	*			
NONE	329.6	288.5	302.5	23.0		
SINGLE	113.3	72.3	86.3	20.8		
TWIN	-62.5	-103.5	-89.5	20.4		
Day 326 to 360	*, **	*, **	*, **			
NONE	189.2^{a}	137.8^{b}	182.5^{a}	23.0		
SINGLE	55.7^{a}	4.3^{b}	48.9^{a}	20.8		
TWIN	20.0^{a}	-31.4^{b}	13.3^{a}	20.4		

[a-c] means within a row without similar superscripts differ ($P < 0.05$).

[*] indicates a linear and [**] indicates a quadratic relationship between number of lambs raised and average daily gain, within treatment within day.

[1] Type III analysis of variance table

[2] SEM = standard error of the mean

Table 7.4 Reproductive performance of hogget ewes provided grain based supplements (100 g/head/d) with either no extract (CON) or 10 mL per head per d of a seaweed (SWO) or a seaweed plus terrestrial plant (SWP) extracts. Values presented here are back-transformed least-squares means from generalized linear models, where the average values of each treatment replicates were used.

	Treatment				
Item [a]	CON	SWO	SWP	SEM	*P*-value
n	4	4	4		
Lambing Date, d from weaning	294.8	287.9	294.4	3.0	0.17
Pregnancy rate, % of ewe exposed to ram	85.0	65.0	90.0	10.2	0.10
Number of Lambs Gestated, count/pregnant ewe	1.30	1.05	1.45	0.17	0.15
Lambs Born Live, count/ pregnant ewe	1.20	0.85	1.10	0.19	0.31
Lambs Weaned, count/pregnant ewe	1.15	0.80	0.95	0.25	0.49
Lambs Lost, count/pregnant ewe	0.15	0.25	0.50	0.27	0.29

[a] These values are based on averages of each paddock (n = 4). Lambing date is based on age from when weaned (14 December 2018). Lambs lost are the number of lambs lost during parturition.

Table 7.5 Plasma urea concentration and plasma total antioxidant status (TAS) of hogget ewe lambs provided grain based supplements (100 g/head/d) with either no extract (CON) or 10 mL per head per d of a seaweed (SWO) or a seaweed plus terrestrial plant (SWP) extracts.

	Treatment				ANOVA	
Item	CON	SWO	SWP	SEm[1]	Term	*P*-value
Plasma TAS, D 53					TRT	0.26
NONE	0.91[ab]	0.99[a]	0.82[b]	0.04	No. lambs	<0.01
SINGLE	0.94[ab]	1.02[a]	0.84[b]	0.05	Day	<0.01
TWIN	0.97[ab]	1.06[a]	0.86[b]	0.04	TRT × No. Lambs	0.49
Plasma TAS, D 125					TRT × Day	<0.01
NONE	0.93[ab]	1.00[a]	0.87[b]	0.04	No. Lambs × Day	<0.01
SINGLE	0.92[ab]	0.99[a]	0.86[b]	0.04	3-way interaction	0.62
TWIN	1.01[ab]	1.09[a]	0.94[b]	0.05		
Plasma TAS, D 280						
NONE	1.33	1.40	1.28	0.07		
SINGLE	1.31	1.38	1.26	0.07		
TWIN	1.26	1.32	1.22	0.07		
Plasma TAS, D 326	*,**	*,**	*,**			
NONE	1.50	1.48	1.52	0.07		
SINGLE	1.30	1.28	1.31	0.05		
TWIN	1.35	1.33	1.36	0.05		
Plasma TAS, D 360	*	*	*			
NONE	1.48	1.48	1.45	0.06		
SINGLE	1.34	1.34	1.31	0.05		
TWIN	1.31	1.30	1.28	0.05		
Plasma Urea, D 53			*,**		TRT	0.45
NONE	5.76[b]	6.07[b]	8.27[a]	0.75	No. lambs	<0.01
SINGLE	6.08[ab]	6.76[a]	5.69[b]	0.50	Day	<0.01
TWIN	6.11	6.03	6.16	0.52	TRT × No. Lambs	0.04
Plasma Urea, D 125			*		TRT × Day	0.81
NONE	6.68[b]	7.82[b]	10.32[a]	0.98	No. Lambs × Day	0.01
SINGLE	7.85	8.38	8.09	0.64	3-way interaction	0.01
TWIN	7.37	7.71	7.78	0.57		
Plasma Urea, D 280						
NONE	11.86	12.21	13.33	1.36		
SINGLE	12.41	10.90	11.68	1.00		
TWIN	10.02	12.67	11.99	1.28		
Plasma Urea, D 326			*			
NONE	10.93	10.59	12.05	0.91		
SINGLE	10.17	9.71	10.37	0.60		
TWIN	9.24[ab]	10.51[a]	8.54[b]	0.61		
Plasma Urea, D 360		*	*			
NONE	6.64[b]	7.56[ab]	8.13[a]	0.49		
SINGLE	6.42	6.08	6.45	0.35		
TWIN	6.04	5.78	6.01	0.31		

[a-b] means in the same row without similar super scripts differ ($P < 0.05$).

* represents a linear and ** represents a quadratic effect between NLR within sampling day and treatment

[1] SEm = standard error of the mean.

Table 7.6 Rumen fermentation characteristics for hogget ewes provided grain based supplements (100 g/head/d) with either no extract (CON) or 10 mL per head per d of a seaweed (SWO) or a seaweed plus terrestrial plant (SWP) extracts. In the event of a treatment (TRT) by Day interaction (Inter.) the values are separated into the different sampling days.

	Treatment				*P*-value		
Item	CON	SWO	SWP	SEm[1]	TRT	Day	Inter.
NH_3, mmol/L					0.17	<0.01	0.03
Day 53	10.2[ab]	10.6[a]	9.0[b]	0.53			
Day 125	5.8	7.2	7.1	0.51			
Day 360	5.2	5.9	6.6	0.53			
Total VFA, mmol/L					0.13	<0.01	0.05
Day 53	99.3[a]	93.9[ab]	83.4[b]	4.3			
Day 125	52.0	59.3	52.7	4.1			
Day 360	58.1[b]	70.0[a]	66.9[ab]	4.5			
A:P, mmol/mmol					0.30	<0.01	0.04
Day 53	3.03[b]	3.26[a]	3.07[ab]	0.08			
Day 125	4.00[b]	4.34[a]	4.25[ab]	0.12			
Day 360	3.59	3.43	3.62	0.10			
Acetate, % of total					0.13	<0.01	0.52
Day 53	63.1	64.5	63.6	0.50			
Day 125	67.6	68.9	68.0	0.53			
Day 360	66.3	66.3	65.8	0.55			
Propionate, % of total					0.53	<0.01	0.06
Day 53	20.8	19.8	20.7	0.50			
Day 125	17.0	16.1	16.1	0.40			
Day 360	18.6	19.4	18.4	0.48			
Butyrate, % of total					0.67	0.03	0.38
Day 53	11.3	11.2	11.1	0.37			
Day 125	11.8	11.9	12.0	0.39			
Day 360	11.6	10.8	11.8	0.40			
Valerate, % of total					0.26	<0.01	<0.01
Day 53	1.34[a]	1.22[b]	1.23[b]	0.04			
Day 125	0.94[a]	0.79[b]	0.93[a]	0.03			
Day 360	1.06[b]	1.17[a]	1.10[ab]	0.04			
Hexanoate, % of total					0.55	<0.01	0.05
Day 53	0.24[a]	0.10[b]	0.16[ab]	0.05			
Day 125	0.07[a]	0.04[b]	0.05[ab]	0.01			
Day 360	0.46	0.45	0.46	0.11			
Iso-butyrate, % of total					0.08	<0.01	0.07
Day 53	1.46	1.42	1.47	0.10			
Day 125	1.22	1.09	1.33	0.09			
Day 360	0.93	0.91	1.17	0.07			
Iso-valerate, % of total					0.03	<0.01	<0.01
Day 53	1.69	1.68	1.68	0.14			
Day 125	1.38[ab]	1.14[b]	1.59[a]	0.13			
Day 360	0.99[b]	0.91[b]	1.35[a]	0.10			

[a-b] means in the same row without similar super scripts differ ($P < 0.05$).

[1] SEm = standard error of the mean.

Table 7.7 Lamb body weight and average daily gain. Lambs were born to hogget ewes provided grain based supplements (100 g/head/d) with either no extract (CON) or 10 mL per head per d of a seaweed (SWO) or a seaweed plus terrestrial plant (SWP) extracts.

	Sex			Number of Lambs			*P*-value[a]	
Item	Ram	Ewe	SEm[b]	Single	Twin	SEm[b]	Sex	No. Lambs
Body Weight, kg								
Birth	4.42	4.14	0.13	4.64	3.92	0.17	0.07	<0.01
Tailing	15.4	14.7	0.45	16.8	13.3	0.58	0.12	<0.01
Weaning	25.5	24.3	0.63	27.2	22.6	0.81	0.08	<0.01
Average Daily gain, g/d								
Birth to tailing	331	308	11.7	363	276	14.4	0.09	<0.01
Tailing to Weaning	299	272	8.98	306	266	11.1	0.01	<0.01
Birth to Weaning	313	297	8.37	335	275	10.7	0.07	<0.01

[a] There were no main effects of treatment detected ($P \geq 0.26$); no two-way and the three-way interaction was not significant ($P \geq 0.21$). Date of birth after the first lamb was born was included as a covariate and explained a significant ($P < 0.05$) amount of variation for lamb body weight at tailing and weaning. No. of Lambs = the number of lambs born (i.e. a single or a twin).

[b] SEm = standard error of the mean.

Table 7.8 Plasma total antioxidant (TAS), plasma urea (PU), whole blood glutathione peroxidase (GPx) activity, and rumen fermentation characteristics of lambs. Samples were taken the day following weaning. Values reported are least-squares means of average values within each replicated paddock (n = 4 per treatment).

	Treatment				
Item	CON	SWE	SWP	SEM	*P*-value
n	4	4	4		
Blood Parameters					
TAS, mmol Eq./L	1.15[b]	1.30[a]	1.32[a]	0.04	0.02
GPx, U/mL	27.2[a]	19.3[b]	18.5[b]	2.02	0.03
PU, mmol/L	4.99[b]	5.73[a]	5.77[a]	0.20	0.04
Rumen Parameters					
A:P, mmol/mmol	3.22[a]	3.39[a]	2.98[b]	0.05	<0.01
Total VFA, mmol/L	41.3[b]	39.7[b]	62.7[a]	2.62	<0.01
Acetate, % of total	64.1[ab]	65.2[a]	63.1[b]	0.49	0.04
Propionate,% of total	20.2[b]	19.4[b]	21.3[a]	0.31	<0.01
Butyrate, % of total	11.2	10.4	11.2	0.49	0.47
Valerate, % of total	1.09[b]	1.09[b]	1.24[a]	0.03	<0.01
Hexanoate, % of total	0.29	0.52	0.48	0.07	0.10
Iso-butyrate, % of total	1.46[a]	1.49[a]	1.23[b]	0.07	0.05
Iso-valerate, % of total	1.58[a]	1.62[a]	1.28[b]	0.09	0.04

[a-c] means within a row without similar superscripts differ ($P < 0.05$).

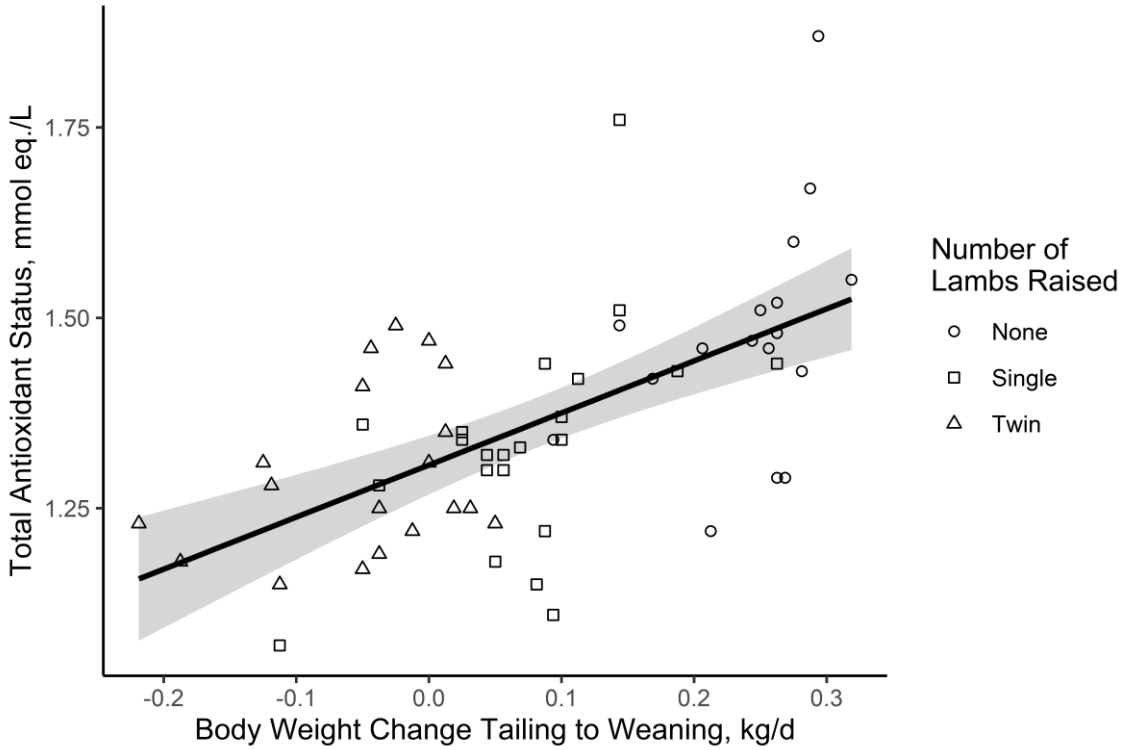

Figure 7.1 As body weight change increases 1-kg, plasma total antioxidant status (TAS) increases by 0.56 mmol equivalence per L. At 0 kg/d day, the TAS was 1.31 (R^2 = 0.36). The shaded band is the 95% confidence interval about the regression line.

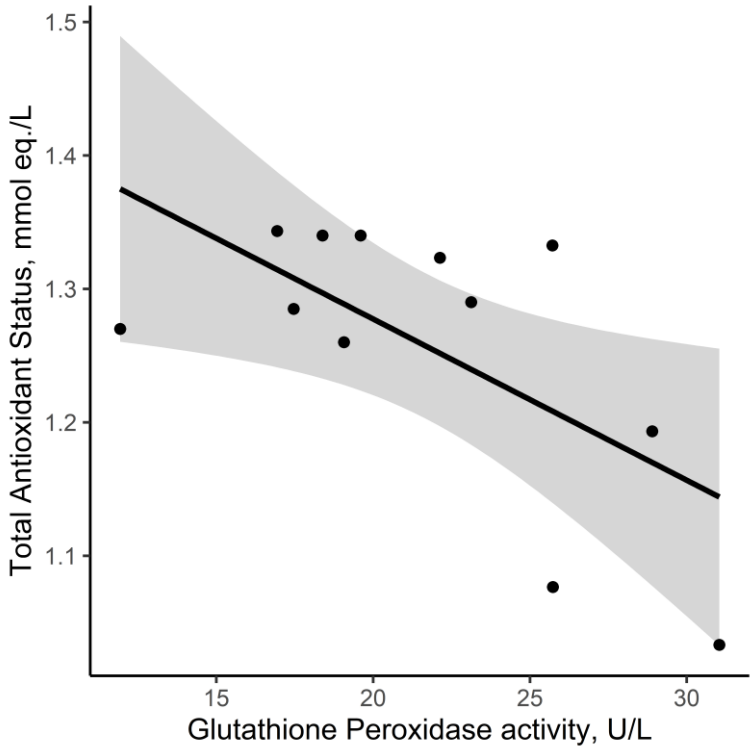

Figure 7.2 Relationship between glutathione peroxidase (GPx) activity and total antioxidant status (TAS) of lambs. The points represent the average values of each replicated paddock and the shaded area is the 95% confidence interval. There was a negative linear relationship determined, so that TAS = GPx × -0.01 + 1.52 (P = 0.03; R^2 = 0.40). This relationship indicates that as antioxidants are depleted, GPx activity is upregulated to account for the additional oxidants.

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Chapter 8

Early life exposure to plant extracts alters dietary preference and grazing behavior of lambs when exposed to a diverse diet.

8.1 Abstract

The objective of this experiment was to determine how early life exposure to plant extracts would influence grazing behavior and dietary preference. This experiment used ram lambs (n = 60; initial body weight = 41.8 ± 3.8 kg, mean ± standard deviation). Their dams were either provided no plant extract (**CON**), a seaweed (*Ecklonia radiata*) extract (10 mL/hd/d; **SWE**), or an extract of seaweed, chicory (*Cichorium intybus*), plantain (*Plantago lanceolata*), lucerne (*Medicago sativa*), and dock (*Rumex obtusifolius*; 10 mL/hd/d; **SWP**). Treatments were provided to the dams starting in late gestation (8 July 2018), through to weaning of the lambs. After weaning (14 December 2018), lambs received the respective treatments of their dams until the initiation of the current experiment (18 February 2019). At the initiation of the current experiment, the lambs were placed into a paddock containing spatially separated strips of ryegrass (*Lolium perenne*), chicory, plantain, lucerne, and dock, of which they received a fresh break, weekly. The behaviors (i.e. grazing, ruminating, or idling) and the location in the paddock (i.e. forage species) of individual lambs were observed, by scan sampling at 5 min intervals on the first day that a new break was provided, for 2 h after sunrise (**AM**) and 2 h before sunset (**PM**). During week 1, SWP had more ($P < 0.05$) scans spent grazing than SWE and CON. Also, during week 1, SWP had a greater ($P < 0.05$) number of grazing bouts and a shorter ($P < 0.05$) grazing bout duration compared with SWE and CON. In week 1, SWP had 78.6% and 167.3% more ($P < 0.05$) proportion of grazing scans spent in chicory than CON and SWE, respectively. Concomitantly, SWP had 33.5% and 59.7% less ($P < 0.05$) grazing scans in ryegrass than CON and SWE, respectively. At the observation weeks 4 and 7, the grazing behavior and dietary preference between treatments was reduced, indicating

learning occurred by CON and SWE. Overall, these results indicate that early life exposure to a plant extract alters dietary preference to the species contained in that extract and also changes grazing behavior, which suggests that the extract provided familiarity to the plants and thereby reduced dietary neophobia.

Keywords: dietary neophobia; grazing ruminants; foraging ecology

8.2 Introduction

Neophobia is the fear of something new. Ruminant livestock experience a significant amount of dietary neophobia, when they are presented with unfamiliar dietary components (Thorhallsdottir et al., 1990; Launchbaugh et al., 1997). Neophobia represents a potential significant economic loss. For instance, when lambs were preconditioned, with their mother, to a grain-based diet early in life, they had greater performance in the feedlot when consuming that diet compared with the naïve lambs (Ortega-Reyes et al., 1992). Additionally, naïve grazing ruminants experiencing neophobia can lead to the degradation of landscapes through overgrazing of familiar foods in the attempt to avoid unfamiliar ones, i.e. selective foraging (Launchbaugh and Howery, 2005). Thus, methods are required to minimize the extent and length of dietary neophobia in order to reduce economic losses and to prevent overgrazing of specific plant species and areas.

Ruminants develop dietary preferences by integrating previous experiences with their current internal state. Dietary exposure begins *in utero*, it continues through exposure through their mother's milk, and ruminants can learn from personal exposure or through observing their peers (Provenza and Balph, 1988). In conjunction with their exposure early in life, postingestive feedback (positive or negative) mechanisms along with specific individual requirements for specific nutrients develop dietary preferences for plant species (Provenza, 1995). Additionally, ruminants learn pre-ingestive cues of plants (i.e. sight, touch, smell, and taste), which they integrate with their earlier experiences (e.g. postingestive feedbacks) and their nutritional requirements in order to construct their daily meals (Favreau-Peigné et

al., 2013). This explains why even young ruminants with dietary experience construct highly variable diets on a day to day basis when choice in dietary components are provided (Atwood et al., 2001). Currently, there is a lack of knowledge on how early life exposure to plant extracts, as opposed to whole diets, might influence dietary preferences and grazing behaviors, later in life. Additionally, it is unknown if dietary preferences are determined by experiences with the plant compounds contained in the extracts or if it is through merely previous experience with unfamiliar flavors. Therefore, the objective of this experiment was to determine if early life experience to plant extracts alters grazing behavior and dietary preference later in life, thereby reducing neophobia in growing lambs. We hypothesize that previous exposure to a plant extract would reduce dietary neophobia of the plants contained in the extract and alter grazing behavior. We further hypothesize that the benefit of previous dietary experience would diminish over time, as the unfamiliar lambs gained experience with the plant species throughout the experiment.

8.3 Materials and Methods

All animal manipulations described were approved prior to the experiment initiation by the Lincoln University Animal Ethics Committee (AEC 2018-25).

8.3.1 Animals and Treatments

Growing ram lambs (n = 60; initial body weight = 41.8 ± 3.8 kg, mean ± standard deviation) were used. The mothers of these lambs were used in a previous experiment (Chapter 6). In brief, the dams were provided a grain based supplement (Sheep Nuts, Reliance Feeds, Canterbury, New Zealand) daily (100 g/hd/d) with either no additional plant extract (**CON**), a seaweed (*Ecklonia radiata*) only extract (**SWE**; 10 mL/hd/d), or a seaweed plus chicory (*Cichorium intybus*), plantain (*Plantago lanceolata*), lucerne (*Medicago sativa*), and dock (*Rumex obtusifolius*; 10 mL/hd/d; **SWP**). These treatments were first applied to the dams during the last third of their gestation (8 July 2018) and continued until weaning (14

December 2018). The respective treatments applied to the dams were provided to the lambs from weaning until this experiment began (18 February 2019). The lambs used in the current experiment were selected to ensure that there were no differences in initial body weight. Lambs within treatment were allocated randomly to replicated paddocks (n = 4 per treatment) which contained 5 lambs each. Lambs received a mark using an aerosol raddle (Sprayline, New Zealand) in order for individual animals to be identified from a distance.

8.3.2 Experimental Paddocks and Grazing Management

There were four, 0.75 ha paddocks (i.e. blocks) used in the current experiment. Forage establishment began by applying glyphosate (4 L/ha). Then the area was plowed, disc harrowed, and rotary tilled. After the soil was prepared, soil cores (15 cm depth) were taken at 20 random locations within each block and composited. The samples were analyzed by a commercial laboratory (Hill Laboratories, Hamilton, New Zealand). Base nutrient levels were found to be 92 kg/ ha of potentially available nitrogen, 30 mg/kg of Resin phosphorus and 172.0 mg/kg potassium, with a pH of 5.6. There were no soil amendments used in this experiment. The blocks were then split into five equal sized strips, with 30 cm between the strips. Within each block, the forage species were randomized to one of the five strips. Planting took place on 2 November 2018 using a seed drill with 7.6 cm row spacing. Each forage species was calibrated to provide a seeding rate of 12, 18, 8, 12, and 13 kg per ha for plantain (cv. Ecotain), ryegrass (cv. One50), chicory (cv. Choice), lucerne (cv. Titan), and dock (hand collected seed of a local ecotype), respectively.

After 65 days (6 January 2019) of growth, the blocks were grazed by placing 50 sheep into each block for 4 days, then sheep were removed, and the blocks were mowed to 3 cm height. This was done to remove the annual weeds that had grown (e.g. fat-hen; *Chenopodium album*). The paddocks were allowed another 42 d of regrowth before the start of the experiment. Each paddock had one replicate per treatment for a total of 4 replicates per treatment. Each replicate provided 100 m^2 per sheep and a

new area was allocated once a week. The areas were allocated using temporary electric net fencing. Once moved, that area was allowed to regrow for 21 d before it was grazed again, each area was grazed twice during the experiment.

8.3.3 Observations and Measurements

Scan sampling was utilized to determine the grazing behavior and dietary preference of lambs (Altmann, 1974; Villalba et al., 2015). During week 1 (18 February), 4 (12 March), and 7 (2 April) of the experiment sheep were observed for 2 hours before sunset (**PM**), as close to their introduction to a new break as possible, and then 2 hours following sunrise (**AM**) the following day. These times were selected as they correspond with the natural circadian rhythm matching the largest grazing bouts of sheep (Champion et al., 1994). Villalba et al. (2015) determined the preference of Angus calves for tall fescue (*Festuca arundinacea*), lucerne, and sainfoin (*Onobrychis viciifolia*) by scan sampling (observing behavior at 5 min intervals) for two hours in the AM only; however, observations were done in the AM and PM to account for any potential diurnal changes in dietary preference between the species, such as what has been reported for sheep offered ryegrass and white-clover (Rutter, 2006). Observers were able to identify individual animals according to their unique markings which were applied using aerosol marker (Sprayline, New Zealand). There was one observer positioned per block of paddocks (i.e. 1 person observing 15 sheep) and care was taken to not disturb the animals' natural behavior. During the observation times, each individual sheep's behavior (i.e. grazing, ruminating, or idling) and their location (i.e. which forage species strip they were in) was recorded every 5 minutes. The proportion of time spent grazing, ruminating, and idle was calculated as the number of scans of them exhibiting this behavior per total number of scans. Grazing bouts were considered to be when a new grazing location (i.e. forage species) was recorded, based on the definition of Gregorini et al. (2006), and grazing bout duration was considered the length of time spent grazing in one particular location (i.e. forage species). Finally, the proportion of grazing time spent in each forage species was also calculated.

Forage measurements were taken on the same days as the observations and were taken prior to introducing the animals to a new break. One quadrat (0.25 m^2) was cut to ground level per forage species per replicate. The fresh weight of the quadrat was recorded and the cut herbage was sorted into three representative sub samples. At random, one sub sample was thrown away, one was used to determine dry matter (**DM**) percentage by weighing fresh weight and oven dried weight (60°C), and one was used to determine botanical composition. The sub sample was sorted based on the morphological parts of the sown species (i.e. reproductive and leaf), weeds, and dead material. Finally, snip cuts were performedby clipping hand grabbed samples in 6 random locations per forage species per replicate. These samples were frozen (-20°C), lyophilized, and then ground to pass through a 1 mm screen (ZM200 Retsch). The ground samples were analyzed for nutritive composition by near infrared spectroscopy (NIRS; FOSS NIRSystems 5000, Maryland, USA). The calibration equations ($R^2 > 0.90$) used by the NIRS were developed prior to the experiment. Calibrations were based on values which were obtained for residual DM (AOAC, 1990; method 930.15), ash (AOAC, 1990; method 942.05), neutral detergent fiber (**NDF**; Van Soest et al., 1991), acid detergent fiber (**ADF**; AOAC, 1990; method 973.18), crude protein (**CP**; by combustion; Variomax CN Analyzer Elementar), water soluble carbohydrates (**WSC**; MAFF, 1986), and DM and organic matter (**OM**) digestibility (DMD and OMD, respectively; Iowerth et al., 1975).

8.3.4 Statistical Analysis

The current experiment was designed as a randomized complete block design. Each main block (i.e. paddock) had one replicate per treatment. All data processing and statistical analysis was conducted using R (R Core Team, 2020, v.3.6.3). All figures were generated using the 'ggplot2' package of R (Wickham, 2016). For all analysis the paddock was a random effect and individual animal was a random effect for measurements taken on the animal. All data was analysed by generalized linear model using the 'glmer' function of the 'lme4' package of R with a Poisson distribution for grazing bouts and a Gamma distribution for all other data (Bates et al., 2015). The model for animal behavior included week

of experiment, treatment, and their interaction and species preference data included week of the experiment, treatment, and all possible two-way, and the three-way interaction as fixed effects. For the animal behavior and species preference data, random effects were observation time (AM and PM), the block, and individual animal. For the forage measurement data, fixed effects were treatment, forage species, experimental week, all possible two-way interactions, and the three-way interaction. For the forage measurement data random effect was the block. Following fitting of the model, an analysis of deviance table using type II Wald Chi-square test was generated using the 'Anova' function of the 'car' package (Fox and Weisberg, 2011). Least-squares means were generated and, based on the significance of the analysis of deviance, pairwise comparisons were conducted using the 'emmeans' function (Lenth, 2018). Differences were considered significant if $P \leq 0.05$.

8.4 Results

The establishment of a homogenous stand of dock was unsuccessful, with dock only represented 14% of the forage mass in the strip whereas weeds constituted 27.4% and dead material constituted 46.4%. Therefore, results are presented, but conclusions as to the benefit of dock will not be drawn.

8.4.1 Botanical Composition and Diet Quality

There were no interactions ($P \geq 0.07$) between the treatments and forage sampling week, so only the main effect of treatment is reported in Table 8.1. There was a tendency ($P = 0.06$) for the forage mass of lucerne provided to the SWE treatment to be greater than the other treatment groups; however, no other tendencies were detected ($P \geq 0.31$) for the main effect of plant extract treatment for the forage mass of the different species provided in the current experiment. For all forage species there was an effect ($P < 0.01$) of sampling week on the forage mass provided to the sheep. For the ryegrass and dock species, week 1 was less ($P < 0.05$) than the other two measurement weeks and week 4 and 7 were not different ($P > 0.05$). Week 1 and 7 forage mass measurements were not different ($P > 0.10$) for the

plantain and lucerne species and both of these weeks had less ($P < 0.05$) forage mass than week 4. The forage mass offered of chicory was the least during week 1, was intermediate in week 7, and greatest during week 4 of the experiment.

There was no treatment by sampling week interactions for the botanical compositions for any of the forage species ($P \geq 0.06$; Table 8.1). Likewise, there were no differences ($P \geq 0.16$) in botanical composition of the forage species offered to each treatment group. However, there were some differences detected in botanical composition by week. For ryegrass, the leaf percentage of the sward was lower ($P < 0.01$) in week 1 than in week 7, while week 4 was numerically intermediate and not different from either week ($P > 0.10$). Accordingly, the proportion of dead material in the ryegrass sward was greater ($P = 0.03$) in week 1 compared with week 7, and again week 4 was numerically intermediate and not different ($P \geq 0.12$) from week 1 or 7. The plantain sward contained the greatest proportion of reproductive stem during week 4 and was different ($P < 0.01$) from week 1 and 7, whereas week 1 and 7 were not different ($P = 0.73$). The proportion of dead material in the plantain sward also tended ($P = 0.09$) to differ by the sampling week. In the lucerne sward, there was a greater ($P < 0.01$) proportion of the sown species during week 4 compared with week 1, week 4 and 7 did not differ ($P = 0.26$), and week 7 was also not different from week 1 ($P = 0.49$). Finally, lucerne during week 4 contained less ($P \leq 0.04$) dead material than both week 1 and 7, and week 1 and 7 were not different ($P = 0.68$).

There was no treatment × week interactions for any of the nutritive composition parameters measured ($P \geq 0.11$; Table 8.2). There was a tendency ($P = 0.08$) for a lower DM content of chicory offered to SWP compared with SWE and also a tendency ($P = 0.07$) for a lower DM content of dock offered to SWP compared with SWE and CON. There were no other main effects ($P \geq 0.18$) of treatment detected for nutritive composition of the forages.

8.4.2 Grazing Behavior

During week 1, SWP lambs spent a larger ($P < 0.05$) proportion of scans grazing than the other treatments (10.5% and 19.1% more than SWE and CON, respectively), while CON and SWE were not different ($P > 0.10$; Table 8.3). The SWE and SWP lambs did not differ ($P > 0.10$) in the proportion of scans spent grazing during week 4; however, they were both greater ($P < 0.05$) than the CON (SWP was 10.7% and SWE was 13.0% greater). During the final week of the experiment, the proportion of scans spent grazing was not different ($P > 0.10$) between the CON and SWP lambs, though SWP and CON were greater ($P < 0.05$; 8.8% and 15.6%, respectively) than the SWE treatment. All treatments had similar grazing behavior patterns across the experimental weeks. All of the treatments had the greatest ($P < 0.05$) proportion of scans spent grazing in week 1, while the proportion of time spent grazing during week 4 and 7 were not different ($P > 0.10$). There was no effect on rumination detected for the main effects of treatment ($P = 0.94$), and experimental week ($P = 0.36$), but there was a tendency ($P = 0.06$) for a treatment by experimental week interaction.

There were no treatment differences ($P > 0.10$) in the proportion of time spent idle during week 1. During week 4, SWE and SWP lambs were not different ($P > 0.10$) for time spent idle, whereas CON spent 76.8% and 58.9% more time idle than SWE and SWP, respectively. The CON treatment spent 49.3% more time idle than SWP ($P < 0.05$), while SWE was intermediate and not different ($P > 0.10$) from either treatment. The CON and SWP spent less proportion of scans idle in week 1 ($P < 0.05$), compared with weeks 4 and 7, and the amount of idle time during week 4 and 7 were not different ($P > 0.10$). The SWE treatment spent the most time idle during week 7, and this was greater ($P < 0.05$) than weeks 1 and 4, which were not different ($P > 0.10$) from each other.

The SWP treatment had more ($P < 0.05$) grazing bouts than SWE (+42.8% and +7.8% for week 1 and 4, respectively) and CON (+44.9% and +33.9% for week 1 and 4, respectively). Accordingly, during these same times, the duration of grazing bouts by SWP lambs were shorter ($P < 0.05$) compared with SWE lambs (-65.1% and 12.0% for week 1 and 4, respectively) and CON (-20.8% and -13.7% for week 1

and 4, respectively). While CON and SWE lambs did not differ ($P > 0.10$) in the number of grazing bouts, SWE lambs had 21.6% more ($P < 0.05$) grazing bouts than CON in week 1. The CON and SWE lambs did not differ in the number or duration of grazing bouts during week 4. In week 7, SWE had the least ($P < 0.05$) number of grazing bouts and the greatest ($P < 0.05$) duration of grazing bouts compared with CON and SWP, and these treatments were not different ($P > 0.10$).

Finally, Pearson's correlations were determined between the average grazing bout duration and the proportion of grazing scans spent in each forage species (Table 8.4). No correlation (r = 0.01; $P = 0.81$) was found between grazing bout duration and proportion of grazing time in ryegrass. There was a negative correlation between chicory (r = -0.19; $P < 0.01$), dock (r = -0.13; $P = 0.01$), and plantain (r = -0.32; $P < 0.01$) and grazing bout duration, whereas lucerne was the only forage species with a positive correlation (r = 0.41; $P < 0.01$).

8.4.3 Dietary Preferences

8.4.3.1 Treatment differences

During week 1, SWP lambs were observed spending the largest proportion of grazing scans in chicory forage strips, being +78.6% and +167.3% greater than CON and SWE lambs, respectively. In addition, CON lambs spent 49.7% more time on chicory strips than SWE (Table 8.5). The CON and SWE treatments were not different ($P > 0.10$) in the proportion of grazing scans grazing ryegrass, but SWP spent a lower (-33.5% to -59.7%) proportion of grazing time in ryegrass compared with the other treatments. There were no other differences between treatments for the other forage species; however, SWP lambs tended ($P = 0.10$) to spend more time grazing plantain compared with the other treatments. During week 4, the only difference between the treatments detected were for SWE lambs spending a lower ($P < 0.05$) proportion of their grazing time in ryegrass compared with CON and SWP. Finally, during week 7 SWP lambs spent a larger ($P < 0.05$) proportion of their grazing time in dock and less ($P < 0.05$) in plantain compared with CON and SWP, which were not different ($P > 0.10$) for all forage species.

8.4.3.2 Within Week Within Treatment Preferences

Preference here is defined as the proportion of grazing time spent in each forage location. The differences between forage species preferences for each treatment during each observation week is shown in Table 8.5. In week 1, CON lambs had a similar ($P > 0.10$) preference for lucerne and ryegrass, prefering ($P < 0.05$) these species to chicory, plantain, and dock, which were all not different ($P > 0.10$). The SWE treatment group had a greater ($P < 0.05$) partial preference for lucerne compared with all other forage species, with a similar ($P > 0.10$) preference for ryegrass and dock, which were preferred ($P < 0.05$) over chicory and plantain. The SWP treatment had a greater preference ($P < 0.05$) for lucerne; however, did not ($P > 0.10$) exhibit a preference between any of the other forage species.

During week 4, SWE and SWP lambs exhibited similar preferences. Both treatments had similar ($P > 0.10$) preferences for chicory and lucerne and they preferred ($P < 0.05$) these species over the other forages. Next, they preferred ($P < 0.05$) plantain over ryegrass and dock, and dock over ($P < 0.05$) ryegrass. The CON lambs also exhibited a similar pattern, with the only difference being that there was no difference ($P > 0.10$) in preference between ryegrass and dock.

In the final week of the experiment, CON lambs showed no difference ($P > 0.10$) in preference for lucerne, chicory, and plantain, however, preferred ($P < 0.05$) these species over ryegrass and dock, and preferred ($P < 0.05$) dock over ryegrass. The SWE treatment had a greater ($P < 0.05$) partial preference for lucerne over all other forage species, had no difference ($P > 0.10$) in preference between chicory and plantain, but preferred ($P < 0.05$) chicory over dock and ryegrass. Additionally, SWE did not ($P > 0.10$) prefer plantain over dock, did prefer ($P < 0.05$) plantain over ryegrass, and did not ($P > 0.10$) exhibit a preference for ryegrass and dock. As with SWE, SWP preferred ($P < 0.05$) lucerne to all other species. The SWP treatment did not exhibit a preference ($P > 0.10$) between chicory, dock, and plantain, but did prefer ($P < 0.05$) chicory and dock over ryegrass. Finally, SWP lambs did not ($P > 0.10$) prefer plantain over ryegrass.

8.4.3.3 Changes in Preference Across Weeks

The changes in preference for each species across the experimental weeks is shown in Table 8.5. The CON treatment preferred ($P < 0.05$) chicory during week 4 more than the other weeks and preferred ($P < 0.05$) chicory during week 7 more than week 1. Chicory was preferred at a similar ($P > 0.10$) level by SWE lambs during weeks 4 and 7 and preferred chicory the least ($P < 0.05$) during week 1. For the SWP treatment, chicory was preferred ($P < 0.05$) during week 4 compared with week 1, while preference for chicory was intermediate during week 7 and similar to all other weeks ($P > 0.10$).

When comparing changes in preference across the experimental weeks, CON lambs preferred ($P < 0.05$) dock during week 1 over week 4, and week 7 was intermediate and did not differ ($P > 0.10$) from the other weeks. The SWE treatment preferred ($P < 0.05$) dock during week 1 over the other two weeks, but preference was not different ($P > 0.10$) for dock for week 4 compared to week 7. Lambs in the SWP treatment preferred dock the least ($P < 0.05$) in week 4, while preference for dock was not different ($P > 0.10$) during week 1 and 7. None of the treatments had differences in preference for lucerne across experimental weeks.

The SWP treatment did not ($P > 0.10$) exhibit changes in preference of the forage species between the experimental weeks. The CON treatment increased their preference for plantain throughout the experiment, so that plantain was preferred ($P < 0.05$): week 7 > week 4 > week 1. Similarly, SWE preferred plantain the least ($P < 0.05$) during week 1, but plantain preference did not ($P > 0.10$) differ between week 4 and 7.

For ryegrass, CON lambs exhibited a greater ($P < 0.05$) preference for ryegrass during week 1, whereas preference for ryegrass was similar ($P > 0.10$) for weeks 4 and 7. The SWE and SWP treatments showed the same pattern for preference of ryegrass. The order of preference for ryegrass was: week 1 > week 7 > week 4 ($P < 0.05$).

8.5 Discussion

It was hypothesized that in utero and early life experience to plant extracts would reduce dietary neophobia and alter grazing behavior when the lambs were provided a choice to graze the plant species contained in the extract later in life. Based on the results of this experiment, this hypothesis is accepted. In addition, it was hypothesized that the dietary preference differences between naïve lambs and lambs with *in utero* and early life exposure would diminish over the course of the experiment as the naïve lambs (i.e. CON and SWE) learned about the plant species offered. This hypothesis was confirmed by this experiment.

8.5.1 Reduced Neophobia by Early Life Exposure

Lambs in the SWP treatment group exhibited much less dietary neophobia compared with CON and SWE. Neophobia is defined as the fear of something new, and dietary neophobia commonly occurs in all classes of livestock (Launchbaugh et al., 1997). For example, naïve ruminants have expressed neophobia to total mixed rations (Hicks et al., 1990), low quality forages (Distel et al., 1994), supplements (Van Tien et al., 1999), forbs (Wallis et al., 2014), and fresh forages (Boland et al., 2011). Thus, dietary neophobia in ruminants can occur for several classes of feeds and forages, which may represent a significant cost to production, through reduced intake and production in all ruminant production systems.

The reduced dietary neophobia by the SWP treatment is evidenced by differences observed in grazing behavior and partial preferences between the forage species, particularly during week 1. The SWP treatment spent the greatest proportion of scans grazing compared with the other treatments. They additionally had a greater number of grazing bouts, which were shorter in duration compared with the other treatments. The greater number of grazing bouts and shorter grazing bout durations indicate that the lambs were switching between feeding patches more frequently, thereby forming a meal (i.e. a grazing event) made up of shorter grazing bouts in a larger number of forage species, indicating less

partial preference between the forage species. Spending more time grazing and having a greater number of grazing bouts of shorter duration in an unfamiliar situation indicates less dietary and situational (i.e. the experimental conditions) neophobia by the SWP lambs.

The SWP treatment displaying reduced dietary neophobia is further corroborated by differences in partial preference for the forage species. During week 1, all treatment groups had a partial preference for lucerne. Additionally, CON and SWE lambs also preferred ryegrass compared to the other forage species, presumably reflecting the fact the lambs were grazed on ryegrass-dominant pastures prior to the start of the experiment. However, SWP did not prefer ryegrass over the other forage species, but rather appeared to compose a more even diet, albeit still displaying a preference for lucerne. Additionally, while CON and SWE lambs spent the least amount of time grazing chicory and plantain compared with the other forage species, SWP incorporated these forage species at a similar level to ryegrass. Finally, while CON and SWE lambs increased their preference for plantain during the weeks following the start of the experiment, the preference for plantain by SWP was unchanged between any of the experimental weeks.

It is proposed that the lowered neophobia by SWP was due to their early life experience to a plant extract derived from chicory, dock, lucerne, plantain, and brown seaweed components. The lambs in the current experiment started to receive exposure to their treatments *in utero* as the CON, SWE, and SWP supplements were provided during the last third of gestation. Further exposure to the extracts occurred after lambing, when their dams received the supplements through to weaning. Finally, experience occurred in early life as the CON, SWE, and SWP treatment supplements were fed from weaning until the beginning of this experiment. Previous experience to dietary factors has been shown to reduce dietary neophobia. Experience to feeds begin *in utero* (Hai et al., 2014), continue through experiences of flavors obtained in mother's milk (Provenza and Balph, 1987), and is further developed by early life experiences (both through dietary exposure and influence of peers; Thorhallsdottir et al., 1990). While this experiment can confirm that early life exposure to plant extracts can reduce neophobia,

further research is required to determine at which stage or stages of exposure (*in utero*, mother's milk, or early life) to the extracts caused this result.

8.5.2 Learning, wanting, and liking

While SWP showed reduced dietary neophobia during week 1, CON and SWE lambs showed the least preference for plantain and chicory. This effect was diminished, so that by week 4 all treatments had a partial preference for chicory and an increased preference for plantain. By week 7 chicory and plantain were incorporated at a significant rate (18-23%) by CON and SWE lambs. This indicates a significant amount of learning. Previous experiences to feeds can increase their acceptance when fed later. For example, when lambs had previous experience with low quality forages they increased their preference for low quality forages later in life, compared with naïve lambs (Distel et al., 1994). Additionally, repeated exposure to novel feeds reduces the neophobic behavior of lambs when they are further exposed to unfamiliar feeds (Launchbaugh et al., 1997; Catanese et al., 2012; Villalba et al., 2012). These examples and the current experiment indicate that learning has a large impact on the dietary preference of ruminants.

The current results suggest that SWP had learned about the flavors of the novel feeds through their previous exposure to the extract, but learning by CON and SWE lambs occurred during the experiment. Learning, as defined by Dukas (2013), is a special type of plasticity that involves internal representations of new information based on current external and internal environments, to ultimately better exploit environmental conditions, which are unique to space and time. With regard to food, learning is driven through post-ingestive feedback systems, such as the reward systems and negative responses (Provenza, 1995; Ginane et al., 2015). Ruminants learn to associate pre-ingestive cues (e.g. sight, smells, and flavor) with post-ingestive feedbacks (both positive and negative), which alters their dietary preferences (Villalba and Provenza, 1996; Favreau et al., 2010a). Dietary 'liking', related to palatability (hedonic pleasure of eating), and wanting, related to postingestive feedbacks, are all

influenced by reward regulation. Liking is driven by changes in the opioid, cannabinoid, and gamma aminobutyric acid (GABA) systems, whereas wanting of a food item is influenced by dopamine. Both liking and wanting can alter the learning component of reward regulation of food intake (Ginane et al., 2015). Thus, during the first experimental weeks, CON and SWE lambs spent a relatively small proportion of their time grazing in chicory (44.0% and 62.6% less than SWP, respectively) and they tended to spend less time grazing in plantain. However, CON and SWE lambs spent 59.7% and 33.5% more time, respectively, grazing ryegrass (a familiar forage) compared with SWP lambs. These results may indicate that CON and SWE lambs were sampling the novel forage herbs in order to learn their post-ingestive feedbacks and spent more time consuming forages that they were familiar with, with the exception of lucerne, which is discussed further below. By week 4 of the experiment, the lambs had adequate experience (i.e. learning) with these forages, so that their relative preference for them increased. Ultimately, this experiment provides further evidence that ruminant livestock learn to overcome neophobia by integrating past experiences develop dietary preferences.

8.5.3 Lambs Prefer Mixed Diets

All lambs mixed their diet and, even with the least preferred species (typically ryegrass with exception for week 1), all forage species were consumed during each of the observation periods. The observations were done when the lambs were first provided a new break, so that if the animals chose they could have composed a monotonous diet out of any of the forage species. Ruminants preferring to mix their diet is common in the literature. When provided simple mixes of grass and legumes, ruminants typically show a partial preference for legumes; however, they prefer to compose a mixed diet, and dietary preferences follow a diurnal pattern (Parsons et al., 1994). Villalba et al. (2015) reported that when cattle were offered a choice between sainfoin, lucerne, and tall fescue, they incorporated all of the forages into their diet, albeit they did exhibit preference for sainfoin > lucerne > tall fescue, rather than only consuming the more palatable legumes. Additionally, lambs had lower levels of markers of

physiological stress when provided choice of dietary components compared with lambs provided a balanced total mixed ration (Villalba et al., 2012; Catanese et al., 2013).

One suggested reason for ruminants mixing their diet is to compose a more balanced diet of primary compounds (e.g. protein and carbohydrates) and for medicines (Provenza, 1996). Due to individual variations of physiology and morphology, no one diet could perfectly meet individual requirements for nutrients (Scott and Provenza, 1999). As ruminant nutritionist formulate diets based on the average animal's requirements, and assuming nutritional requirements follow a normal distribution, approximately 50% of animals will be under and over supplied nutrients, with a small number perfectly meeting their requirements (Scott and Provenza, 1999). Thus, it is believed that providing dietary choice to ruminants will allow them to self-select a diet that is balanced to their individual requirements. Accordingly, many experiments that have offered ruminants choice reported greater feed conversion efficiency increments compared with monotony (Nocek et al., 1986; Atwood et al., 2001; Yurtseven and Görgülü, 2004; Atwood et al., 2006). Providing dietary choice also allows animals to mix diets in order to receive medicinal properties (e.g. antioxidant, antibiotic, anthelmintic, etc.) of plant secondary compounds while not overstepping thresholds of toxicity (Beck and Gregorini, 2020). Dietary diversity may allow animals to select diets, which better meets their individual requirements for nutrients and medicines, which may explain why animals prefer a diverse instead of a monotonous diet.

It has been suggested that ruminants may seek a diverse diet over a monotonous diet more for hedonic wellbeing rather than for functional purposes (i.e. a balanced diet). This suggestion was based on observations that sheep mixed their diet regardless of post-ingestive consequence (Favreau et al., 2010b). It is known that pre-ingestive cues (e.g. flavor) can provide hedonic wellbeing independent of post-ingestive feedbacks (Ginane et al., 2011). Accordingly, lambs fed a mixed diet of lucerne and barley (75:25) with either a choice between the unflavored diet or flavored with sweet, umami, or bitter had greater intake and tended to have greater performance compared with treatments fed only one of the flavor profiles (Villalba et al., 2011). As the diet was the same for the treatments, the post-ingestive

consequences were likely the same for all of the flavored diet, so that the increased intake by providing flavor diversity was likely due to the stimulation provided by flavor diversity. Thus, ruminants may prefer a diverse diet because of the stimulation that it provides.

8.5.4 Preference for Lucerne

All treatment groups preferred lucerne during every observation week. This can be seen by the differences in proportion of grazing scans spent in lucerne. This is further corroborated by the correlation between the average grazing bout duration and the proportion of grazing time spent in each species (Table 8.4). While ryegrass did not have a correlation (r = 0.01; *P* = 0.81) and the other forage species having a negative correlation, lucerne had a positive correlation, indicating that as lambs spent a greater proportion of their grazing time consuming lucerne, they also extended their grazing bouts. The preference sheep and other ruminants have for legumes is well documented. One review concluded that the preference of legumes compared with grasses is consistent across livestock species and physiological states, with a partial preference for legumes (~70% of diet) compared with grasses (Rutter, 2006). One proposed theory is that legumes allow ruminants to maximize energy intake rates, with legumes typically having 45% to 60% greater intake rates compared with grasses (Chapman et al., 2007). Regardless of the reasoning, the lambs in the current experiment had a greater partial preference for lucerne compared with the other forage species utilized in the current experiment, which corroborates previous experiments.

The sheep immediately incorporated lucerne into their diet and apparently did not experience neophobia when eating lucerne. This lack of dietary neophobia by ruminants for lucerne has been reported before. Boland et al. (2011) reported that inexperienced cattle spent 78% of their time grazing lucerne and spent less, 72% of grazing time, consuming lucerne after they had experience with lucerne. The lack of neophobia of the lambs to lucerne may be caused by lucerne's high-palatability and its sensory characteristics. For example, some sensory characteristic (e.g. smell, taste, texture, etc.) have

hedonic value (pleasure inducing) outside of their post-ingestive feedback (Favreau-Peigné et al., 2013). In contrast, both plantain and chicory contain plant secondary compounds, such as condensed tannins (Rumball et al., 1997). Condensed tannins are associated with astringency and a bitter taste (Lesschaeve and Noble, 2005), which is expected to have a lower hedonic value (Ginane et al., 2011), so that the initial preference for these forage herbs was much less compared with lucerne during week 1 for CON and SWE. As such, the acceptance of a novel feed by naïve animals may be an indicator of pre-ingestive hedonic value in feeds (Favreau-Peigné et al., 2013).

8.6 Implications

The current experiment has several implications. Firstly, ruminants prefer a diverse diet over a monotonous one. Secondly, plant extracts may provide a means for grazing managers to provide prior experience to a particular feed to reduce neophobia of naïve animals for either targeted grazing of weeds or to increase utilization of typically under-utilized feed resources in a landscape. Finally, naïve ruminants will eventually incorporate novel feeds into their diet, albeit after a period of dietary neophobia.

Table 8.1 Initial forage mass and botanical composition from forage species sown into spatially separated strips and provided to lambs. Lambs were either exposed to no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP) in their early life. Dietary treatments were first applied to their dams during the last third of gestation (8 July 2018), were continued to be applied to their dams through weaning (14 December 2018), and were given to lambs after weaning until the start of the experiment (18 February 2019).

	Treatment				*P*-value[1]		
Strip	CON	SWE	SWP	SEm[2]	Treat.	Week	Inter.
Ryegrass, kg DM/ha	5,849.5	5,498.0	6,100.8	425.0	0.31	<0.01	0.35
Leaf, % of Sward	38.0	31.9	34.7	4.1	0.37	<0.01	0.25
Repro, % of Sward	0.1	0.0	0.1	0.1	0.57	0.16	0.73
Weed, % of Sward	2.0	1.0	6.9	3.4	0.38	0.56	0.51
Dead, % of Sward	55.8	63.5	55.1	5.8	0.35	0.03	0.32
Chicory, kg DM/ha	3,222.0	2,947.1	3,208.8	262.5	0.34	<0.01	0.06
Leaf, % of Sward	58.4	63.4	67.5	4.3	0.22	0.13	0.99
Repro, % of Sward	0.0	0.0	0.0	0.0	---	---	---
Weed, % of Sward	4.4	1.4	0.9	2.2	0.16	0.79	0.19
Dead, % of Sward	35.2	34.2	30.7	4.3	0.71	0.20	0.96
Plantain, kg DM/ha	3,513.7	3,461.2	3652.6	324.7	0.84	<0.01	0.66
Leaf, % of Sward	51.7	54.6	54.6	4.3	0.81	0.44	0.54
Repro, % of Sward	3.2	2.9	5.3	1.3	0.36	<0.01	0.75
Weed, % of Sward	3.9	2.4	2.3	2.0	0.70	0.57	0.31
Dead, % of Sward	40.0	39.2	36.4	4.3	0.82	0.09	0.87
Lucerne, kg DM/ha	3,893.3	3,951.6	4,545.4	282.1	0.06	<0.01	0.07
Leaf, % of Sward	58.0	61.0	66.5	5.4	0.35	0.03	0.49
Repro, % of Sward	0.0	0.0	0.0	0.0	---	---	---
Weed, % of Sward	1.8	1.3	1.3	0.7	0.66	0.19	0.35
Dead, % of Sward	34.8	33.7	29.0	4.4	0.65	<0.01	0.20
Dock, kg DM/ha	3,289.0	3,174.5	3,173.2	265.4	0.91	<0.01	0.91
Leaf, % of Sward	12.9	13.6	15.5	4.7	0.91	0.73	0.06
Repro, % of Sward	2.5	2.7	2.6	1.3	0.99	<0.01	0.54
Weed, % of Sward	27.3	31.4	23.4	5.5	0.57	0.01	0.12
Dead, % of Sward	48.4	41.9	48.9	7.0	0.63	0.01	0.40

[1] Treat. = main effect of treatment; Inter. = the treatment × week interaction

[2] SEm = standard error of the mean

Table 8.2 Dry matter percentage of quadrat cuts and nutritive value of snip cuts taken from forage species sown into spatially separated strips and provided to lambs. Lambs were either exposed to no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP) in their early life. Dietary treatments were first applied to their dams during the last third of gestation (8 July 2018), were continued to be applied to their dams through weaning (14 December 2018), and were given to lambs after weaning until the start of the experiment (18 February 2019).

	Treatment				*P*-value[1]		
Strip	CON	SWE	SWP	SEm[2]	Treat.	Week	Inter.
Ryegrass DM, % as-is	32.5	33.4	35.6	1.4	0.11	<0.01	0.61
CP, % DM	19.5	18.9	19.2	1.0	0.87	0.19	0.59
NDF, % DM	52.0	51.9	51.6	1.1	0.97	0.09	0.49
ADF, % DM	28.3	27.8	28.4	0.7	0.79	<0.01	0.68
WSC, % DM	8.1	8.7	9.3	0.6	0.31	<0.01	0.11
DMD, % DM	69.7	70.4	70.1	1.2	0.91	<0.01	0.72
Chicory DM, % as-is	15.5	16.2	13.0	1.4	0.08	0.03	0.83
CP, % DM	18.7	18.4	18.1	0.8	0.76	<0.01	0.15
NDF, % DM	18.1	17.9	18.3	0.4	0.64	<0.01	0.32
ADF, % DM	13.7	14.4	14.2	0.4	0.39	<0.01	0.11
WSC, % DM	20.6	18.5	19.2	1.0	0.28	<0.01	0.72
DMD, % DM	84.0	83.2	84.1	0.4	0.18	<0.01	0.77
Plantain DM, % as-is	19.0	21.9	18.3	2.0	0.31	0.10	0.27
CP, % DM	15.5	17.1	15.7	0.9	0.32	<0.01	0.48
NDF, % DM	20.2	19.1	19.2	0.7	0.51	<0.01	0.32
ADF, % DM	20.7	19.8	20.1	0.3	0.18	<0.01	0.14
WSC, % DM	17.3	18.0	17.5	0.8	0.79	<0.01	0.53
DMD, % DM	77.9	79.6	78.3	0.7	0.20	<0.01	0.76
Lucerne DM, % as-is	30.0	29.3	26.9	1.9	0.38	0.61	0.40
CP, % DM	22.8	22.7	23.5	0.6	0.48	<0.01	0.21
NDF, % DM	34.4	35.5	34.5	1.4	0.75	<0.01	0.24
ADF, % DM	29.0	29.5	28.9	1.0	0.88	<0.01	0.34
WSC, % DM	7.3	7.0	6.5	0.4	0.31	<0.01	0.23
DMD, % DM	66.4	65.4	66.3	1.3	0.78	<0.01	0.33
Dock DM, % as-is	30.5	31.3	26.6	1.8	0.07	<0.01	0.43
CP, % DM	21.4	19.7	19.1	1.5	0.31	<0.01	0.49
NDF, % DM	22.8	22.0	23.0	0.7	0.42	0.59	0.55
ADF, % DM	16.6	16.1	18.0	1.7	0.39	<0.01	0.78
WSC, % DM	12.3	13.2	12.6	0.8	0.51	0.01	0.96
DMD, % DM	70.3	69.7	69.1	1.3	0.73	<0.01	0.20

[1] Treat. = main effect of treatment; Inter. = the treatment × week interaction

[2] SEm = standard error of the mean

Table 8.3 The total proportion of time spent grazing, ruminating, or idling, and the average time spent grazing one species before changing their behavior, of sheep finished on a diverse paddock. Paddocks were spatially separated strips of ryegrass, chicory, plantain, lucerne, and dock planted. Observations were done for 2 hours before sunset (PM) during the first day that sheep were provided a new break of forage and 2 hours after sunrise (AM) the following morning, during the first day that sheep were provided a new break of forage, during the first, fourth, and seventh week that sheep were allocated to the paddocks. Sheep were either exposed to no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP) in their early life. Dietary treatments were first applied to their dams during the last third of gestation (8 July 2018), were continued to be applied to their dams through weaning (14 December 2018), and were given to lambs after weaning until the start of the experiment (18 February 2019).

	Treatment				Analysis of Deviance[2]		
Item	CON	SWE	SWP	SEM[1]	Variable	Fixed Effect	*P*
Week 1					Grazing:	Treatment	0.03
Grazing, % of scans	65.9^{bA}	71.0^{bA}	78.5^{aA}	9.8		Week	0.02
Ruminating, % of scans	18.7	15.7	11.5	7.1		Treatment × Week	<0.01
Idling, % of scans	10.6^{B}	10.0^{B}	7.4^{B}	1.5	Ruminating:	Treatment	0.94
Grazing Bouts, count	6.9^{b}	7.0^{b}	10.0^{a}	5.0		Week	0.36
Grazing bout dur., min	12.0^{bA}	14.6^{aA}	9.5^{cA}	1.0		Treatment × Week	0.06
Week 4					Idling:	Treatment	0.03
Grazing, % of scans	49.4^{bB}	55.8^{aAB}	54.7^{aB}	5.1		Week	<0.01
Ruminating, % of scans	11.8	12.5	12.4	8.2		Treatment × Week	0.04
Idling, % of scans	26.7^{aA}	15.1^{bB}	16.8^{bA}	3.8	Grazing Bouts:	Treatment	<0.01
Grazing Bouts, count	6.2^{b}	7.7^{b}	8.3^{a}	5.0		Week	0.92
Grazing bout dur., min	10.2^{aB}	10.0^{aB}	8.8^{bB}	0.6		Treatment × Week	<0.01
Week 7					Grazing Bout Dur:	Treatment	<0.01
Grazing, % of scans	54.9^{aB}	47.5^{bB}	51.7^{aB}	5.0		Week	<0.01
Ruminating, % of scans	5.8	5.9	6.3	2.2		Treatment × Week	<0.01
Idling, % of scans	25.2^{abA}	33.6^{aA}	22.5^{bA}	4.8			
Grazing Bouts, count	9.1^{a}	6.9^{b}	9.0^{a}	4.6			
Grazing bout dur., min	7.5^{bC}	8.8^{aB}	7.6^{bB}	0.5			

$^{a-c}$ values within a row without similar superscript differ ($P < 0.05$).

$^{A-C}$ values within treatment within forage species across week without similar superscripts differ ($P < 0.05$).

[1] SE_m = standard error of the mean. The value reported is the largest standard error of the mean for that forage species during the observation time.

[2] The analysis of deviance utilizes a Wald Chi-square test.

Table 8.4 Pearson's correlation (r) between the average grazing bout duration (minutes) and the proportion of their grazing time in the respective forage species.

Forage Species	r	*P*-value
Chicory	-0.19	<0.01
Dock	-0.13	0.01
Lucerne	0.41	<0.01
Plantain	-0.32	<0.01
Ryegrass	0.01	0.81

Table 8.5 The proportion of grazing time that lambs spent grazing chicory, dock, lucerne, plantain, and ryegrass. The plant species were sown in spatially separated strips. Observations were done for 2 hours before sunset during the first day that sheep were provided a new break of forage and 2 hours after sunrise the following morning (time was a random effect), during the first, fourth, and seventh week that sheep were allocated to the paddocks. Lambs were either exposed to no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP) in their early life. Dietary treatments were first applied to their dams during the last third of gestation (8 July 2018), were continued to be applied to their dams through weaning (14 December 2018), and were given to lambs after weaning until the start of the experiment (18 February 2019).

	Treatments				
Item[1]	CON	SWE	SWP	Analysis of Deviance *P*-value[2]	
Week 1				Treat.	0.99
Chicory	11.03 (1.74)^{bxC}	7.37 (1.17)^{cyB}	19.70 (3.12)^{axbB}	Week	0.35
Dock	14.43 (2.29)^{xA}	18.25 (2.89)^{xA}	18.81 (2.98)^{xA}	Spec.	<0.01
Lucerne	37.45 (5.93)^{w}	41.74 (6.61)^{w}	31.19 (4.94)^{w}	Treat. x Week	0.97
Plantain	9.26 (1.47)^{xC}	9.38 (1.49)^{yB}	12.88 (2.04)^{x}	Treat. x Spec.	0.05
Ryegrass	27.82 (4.41)^{awA}	23.26 (3.68)^{abxA}	17.42 (2.76)^{bxA}	Week x Spec.	<0.01
Week 4				Treat. x Week x Spec.	<0.01
Chicory	40.83 (6.46)^{wA}	29.41 (4.66)^{wA}	31.05 (4.92)^{wA}		
Dock	8.72 (1.38)^{yB}	11.50 (1.82)^{yB}	10.12 (1.60)^{yB}		
Lucerne	29.44 (4.66)^{w}	36.28 (5.74)^{w}	36.62 (5.80)^{w}		
Plantain	15.29 (2.42)^{xB}	19.83 (3.14)^{xA}	17.96 (2.84)^{x}		
Ryegrass	5.71 (0.91)^{ayB}	2.98 (0.47)^{bzC}	4.25 (0.67)^{abzC}		
Week 7					
Chicory	22.65 (3.68)^{wB}	20.58 (3.26)^{xA}	21.18 (3.35)^{xAB}		
Dock	13.53 (2.20)^{bxAB}	12.15 (1.92)^{byzB}	21.25 (3.36)^{axA}		
Lucerne	32.30 (5.25)^{w}	39.48 (6.25)^{w}	33.36 (5.28)^{w}		
Plantain	23.11 (3.75)^{awA}	17.93 (2.84)^{abxyA}	14.52 (2.30)^{bxy}		
Ryegrass	8.41 (1.37)^{yB}	9.87 (1.56)^{zB}	9.69 (1.54)^{yB}		

$^{a-c}$ values within a row without similar superscript differ ($P < 0.05$).
$^{w-z}$ values within a column, within week without similar superscripts differ ($P < 0.05$).
$^{A-C}$ values within treatment within forage species across week without similar superscripts differ ($P < 0.05$).
[1] Values reported are proportion of grazing time and the standard error of the mean in parenthesis.
[2] The analysis of deviance utilizes a Wald Chi-square test.

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Chapter 9

Providing a plant extract to dams reduces dietary neophobia of grazing lambs.

9.1 Abstract

The objective of this experiment was to determine how previous exposure to plant extracts can alter grazing behavior and reduce dietary neophobia of naïve growing lambs. This experiment used 18 rams (33.5 ± 3.1 kg body weight; BW; mean ± standard deviation) and 18 ewes (30.1 ± 4.0 kg BW). All of their dams were provided a grain-based supplement daily (100 g/hd/d). The supplements contained either no additional plant extract (CON), a seaweed (*Ecklonia radiata*) only extract (SWE; 10 mL/hd/d; Agrisea Ltd; Paeroa, New Zealand), or a seaweed plus chicory (*Cichorium intybus* L.), plantain (*Plantago lanceolata* L.), lucerne (*Medicago sativa* L.), and dock (*Rumex obtusifolius* L.) extract (SWP; 10 mL/hd/d; Agrisea Ltd; Paeroa, New Zealand). The treatment supplements provided to the hogget ewes were fed in a manor to preclude access to the lambs. Lambs were grouped by sex and treatment and allocated randomly to one of 12 paddocks (n = 4 per treatment) with 3 lambs per paddock, 35 days after weaning. All paddocks contained equal land areas of spatially strips which were sown the previous year to ryegrass (*Lolium perenne* L.), chicory, plantain, lucerne, and dock. The behavior (grazing, ruminating, or idling) and the location in the paddock of each lamb was recorded, by scan sampling at 5 minute intervals from sunrise (0600 h) to sunset (2120 h). There were no treatment differences detected for the forage mass of the species ($P \geq 0.14$), nor in botanical composition ($P \geq 0.27$). The SWP lambs had a greater ($P < 0.05$) partial preference for chicory in the AM and PM, compared with CON and SWE lambs. The SWP lambs also had a greater ($P < 0.05$) partial preference for lucerne than CON lambs in the AM and greater ($P < 0.05$) partial preference for lucerne than CON and SWE lambs in the PM. These results suggest that exposure to forages can occur through the maternal transmission of plant characteristics, from the *in utero* or

maternal milk exposure. The dietary experience gained from their mothers was retained by the lambs even 35 days after weaning.

Key words: Dietary neophobia; forage preference; foraging ecology; ruminant livestock

9.2 Introduction

Dietary neophobia is the fear of unfamiliar foods. Ruminants experience a considerable amount of dietary neophobia when first introduced to a new feed (Thorhallsdottir et al., 1990; Launchbaugh et al., 1997). This phenomenon can have several negative implications. For example, dry matter intake (DMI) is reduced by dietary neophobia, which can in turn hinders animal performance (Hicks et al., 1990). Additionally, animals introduced to an unfamiliar landscape may degrade the area by overgrazing familiar forages and underutilizing unfamiliar forages (Launchbaugh and Howery, 2005). Both of these eventualities can reduce the profitability of ruminant production systems, with the later having the potential to harm the future productivity of the landscape. Therefore, it is important to farm productivity to negate the detrimental impacts of dietary neophobia by familiarizing animals with the food items they may encounter.

Dietary experience begins *in utero*. For instance, Hai et al. (2014) fed *Chromonaela odorata* to late gestating goats, which increased intake of that plant by their kids compared with goat kids whose dam did not receive the plant. Further, volatile plant characteristics, such as those that impart smells and flavors, can be incorporated into milk (Besle et al., 2010; Di Trana et al., 2015). It is therefore believed that mothers milk is another means for the maternal transmission of dietary experience (Provenza and Balph, 1988). Early life experiences provide memories associated with post-ingestive feedback (positive or negative; Provenza, 1995), which ruminants associate with that dietary constituent's sensorial properties (sight, touch, smell, and taste) as pre-ingestive cues (Favreau-Peigné et al., 2013). These sources of dietary exposure are integrated with the animal's current internal state, to develop short-term and ultimately long-term dietary preferences (Provenza, 1995).

Previous work in our laboratory found that by providing early life exposure to forage species by providing a plant extract reduced dietary neophobia of chicory (*Chicorium intybus* L.) and altered grazing behavior in growing lambs when first introduced to a diverse diet (Chapter 8). The lambs were exposed to the extract *in utero* and in early life*,* as their dams were provided the extract during the last third of gestation through to weaning; however, following weaning the lambs themselves were provided the extract until the start of the experiment. Thus, we were unable to determine if the reduced dietary neophobia was due to the exposure to the extract through maternal transmission or during early life experience post weaning. Thus, the objective of the current experiment was to determine if providing a plant extract to dams during gestation through to weaning could reduce dietary neophobia of the lambs, even after a relatively long period following weaning. We hypothesized that maternal transmission of plant characteristics —provided by an aqueous plant extract— would provide prior experience to novel forages, subsequently reducing dietary neophobia.

9.3 Materials and Methods

All animal manipulations described were approved prior to the experiment initiation by the Lincoln University Animal Ethics Committee (AEC 2018-25).

9.3.1 Animals, Treatments, and Observations

This experiment was conducted on the 14 January 2020 (35 days after weaning) and used Coopworth lambs (103 ± 8 days old), 18 rams (33.5 ± 3.1 kg body weight; BW; mean ± standard deviation) and 18 ewes (30.1 ± 4.0 kg BW). The lambs were born to hogget ewes and management from their weaning, to weaning the lambs in the current experiment has been described previously (Chapter 7). Briefly, the hogget ewes were all provided a grain-based supplement daily (100 g/hd/d; Sheep Nuts; Reliance Feed Mills; Rolleston, New Zealand). The supplements contained either no additional plant extract (CON), a seaweed (*Ecklonia radiata*) only extract (SWE; 10 mL/hd/d), or a seaweed plus chicory,

plantain (*Plantago lanceolata* L.), lucerne (*Medicago sativa* L.), and dock (*Rumex obtusifolius* L.) extract (SWP; 10 mL/hd/d). The extracts were created according to the proprietary methods of Agrisea Ltd (Paeroa, New Zealand). These treatments were fed to the hogget ewes from their weaning until their lambs were weaned and were fed off the ground in troughs that precluded their lambs from accessing the supplement. Thus, the only exposure the lambs would have to the plant extracts would be transmitted from the dam. The SWE treatment group can be considered a positive control, so that we can conclude if any differences in behavior or dietary preferences was merely because of previous exposure to any plant extract. This may be possible because it has been shown that repeated exposure to novel plants reduces the neophobia when further novel feeds are fed (Launchbaugh et al., 1997; Villalba et al., 2012).

The paddocks used in the current experiment consisted of spatially separated strips of equal sizes, which were sown with either chicory, plantain, lucerne, dock, and perennial ryegrass (*Lolium perenne* L.). Paddocks were sown on 2 November 2018 and establishment was described previously (Chapter 8). The lambs were grouped by sex and treatment and randomly allocated to one of 12 paddocks (n = 4 per treatment), with 3 lambs per paddock. A minimum of three lambs per paddock is needed for the animals to display normal grazing behavior (Penning et al., 1993). The stocking density was set at 250 m^2/lamb to ensure that the lambs had adequate forage to compose their entire diet for the observation day of one forage species if they chose. All lambs were placed into a ryegrass paddock on 13 January 2020 according to their replicate, which was near to their respective experimental paddock. The following morning (0530 h) the lambs were shifted into their experimental paddock.

Grazing behavior and dietary preferences of lambs were determined using scan sampling (Altmann, 1974; Villalba et al., 2015). Scan sampling was done in 5 minute intervals, from sunrise (0600 h) to sunset (2120 h), and the behavior, either grazing, ruminating, or idling, and their location (i.e. forage species strip) was recorded. Villalba et al. (2015) likewise used 5 min scan intervals to estimate grazing behavior and dietary preference of cattle and sheep, respectively. A trained observer was

assigned 4 paddocks (1 person per 12 sheep), so that they recorded the individual behavior and location for 12 animals. All lambs had a unique marking using an aerosol marker (Sprayline, New Zealand), so that they could be identified by observers from a distance.

The recorded data set was separated into morning (AM; 0600–1200 h; 360 minutes) and afternoon (PM; 1200–2120 h; 560 minutes). Grazing behavior was calculated as a proportion of total observation time (i.e. number of scans) spent grazing, ruminating, and idling. A grazing bout is defined as grazing within a specific patch (i.e. a cluster of feeding stations) and that a grazing event is composed of a cluster of grazing bouts (Gregorini et al., 2006). We considered a patch to be a forage species strip. Grazing bouts were considered to be when a new grazing location (i.e. forage species) was recorded and is expressed as a count. The grazing bout duration was estimated by calculating the average duration the lamb spent grazing in a specific forage species before changing location or behavior. Finally, percentage of time spent grazing in each forage species was calculated.

9.3.2 Forage Sampling and Analysis

All forage samples were collected on 13 January 2020. In each strip, of each paddock, a quadrat (i.e. 0.25 m^2 area) was randomly selected and cut to ground level using electric hand clippers. The clipped forage was collected and dried at 60°C for 7 days. The dry weight for each quadrat cut was weighed, and forage mass was determined by extrapolating the dry quadrat weight to kg of forage dry matter (DM) per ha. Additionally, in each forage strip of each paddock, snip-cut sampling was done at 10 random locations by cutting a hand grab sample to ground level. The snip-cuts were thoroughly mixed and then separated into 3 equal sized sub-samples. Of these three sub-samples, one was used to determine the DM percent, another the botanical composition, and the last was used to determine the chemical composition of the herbage. At random, the fresh weight of one of the snip-cut sub-samples was recorded, the sample was dried for 7 days at 60°C, and after recording the dry weight of the sample was recorded, and DM percent was calculated. The snip-cut subsamples used to determine botanical

composition was sorted, according the sown species leaf, sown species stem (chicory only), sown species reproductive, weeds, and dead material. The botanical composition of the sorted grass was then determined based on the weight of the respective component and the total weight of the botanical components. The final sub-sample from the snip-cuts was frozen at -20°C, was lyophilized, and then ground by a centrifugal mill (ZM200; Retsch GmbH; Haan, Germany) to pass through a 1 mm screen.

The ground forage samples were analyzed for nutritive composition using near infrared spectroscopy (NIRS; FOSS NIRSystems 5000; Maryland, USA) using established calibration equations (R^2 > 0.90). The calibrations equations were done by wet chemistry for residual DM, ash, and acid detergent fiber (ADF; AOAC, 1990; methods 930.15, 942.05, and 973.18, respectively), neutral detergent fiber (NDF; Van Soest et al., 1991), crude protein (CP) by combustion (Vario Max CN Analyzer; Elementar Analysensysteme GmbH; Langenselbold, Germany), and DM digestibility (DMD; (Iowerth et al., 1975).

9.3.3 Statistical Analysis

This experiment was a completely randomized design. The R software was used for all statistical analysis (R Core Team, 2020; v.3.6.3). Several generalized linear mixed models, with different distributions were explored. A Gaussian (i.e. normal) distribution was found to be the most appropriate based on the Quantile-Quantile plots of model residuals. Accordingly, a mixed effects model was implemented for all of the variables analysis using the 'lmer' function of the 'lme4' package (Bates et al., 2015). As the observational units was on the animal level and the experimental units was the paddock replicates, individual animal was used as a random effect. For grazing behavior variable, proportion of time grazing, ruminating, and idling, grazing bouts, and grazing bout duration, fixed effects were treatment, time (AM and PM), and the treatment × time interaction. For the proportion of time spent grazing each forage species fixed effects included treatment, forage species, time, and all possible two- and the three-way interactions. A type III analysis of variance (ANOVA) table, with Satterthwaitte's degrees of freedom approximation was generated using the 'anova' of the base R software (R Core Team, 2020; v.3.6.3).

Least-squares means were generated and, depending on the significance of the ANOVA, mean separation was done using pairwise t-tests with the 'emmeans' function (Lenth, 2018). For the grazing behavior variables, all treatment × time contrasts were generated. For the grazing preference data, treatment × time within forage species and contrasts testing forage species within treatment within time, were generated. Statistical significance was declared at $P \leq 0.05$.

9.4 Results

The strip sown to dock was, on average, 52% weeds predominantly monocotyledon species, such as perennial ryegrass and *Poa annua*, with some dicotyledon species, such as white clover (*Trifolium repens*) and mallow (*Malva spp.*). This indicates that while lambs spent a considerable amount of time grazing in dock, they were likely consuming the non-sown species. Accordingly, the results of dock treatment are reported, but not used to discuss treatment effects.

There were no differences ($P \geq 0.14$) between the treatments for forage mass nor the botanical composition for any of the forage species (Table 9.1). There were no treatment differences detected for the nutritive quality of the forage species offered to the lambs (Table 9.2).

There tended ($P = 0.09$) to be a main effect of treatment where SWE lambs spent a greater proportion of time grazing compare with CON and SWP lambs (Table 9.3). There were no other main effects ($P > 0.10$) of treatment or treatment × time interactions for proportion of time grazing, ruminating, or idling, however there was a significant ($P < 0.05$) main effect of time. All lambs spent more ($P < 0.05$) time grazing and ruminating, and less ($P < 0.05$) time idling in the AM than PM. There was a treatment × time interaction ($P < 0.05$) for the number of grazing bouts and grazing bout duration. The SWE lambs had a similar ($P > 0.10$) number of grazing bout is in AM compared to the PM, whereas the other treatments had less ($P < 0.05$) grazing bouts in the AM compared with the PM. The CON lambs had a longer ($P < 0.05$) grazing bout duration than SWE lambs in the AM and there were no other ($P > 0.10$) differences between grazing bout duration.

There were no significant (P = 1.00) main effects for time or treatment (Table 9.4). There was at treatment × forage species × time interaction (P < 0.01) reflecting the fact SWE and CON lambs had similar preference for the forage species in both AM and PM. Both SWE and CON lambs preferred (P < 0.05) dock compared with all the other species in the AM (Table 9.5). The CON lambs preferred ryegrass more (P < 0.05) than chicory and lucerne, while plantain was intermediate and not different (P > 0.10) from any species except for dock. For the SWE lambs, the rest of the forage species were not different (P > 0.10) and were less preferred (P < 0.05) than dock. In the AM, the proportion of grazing time spent in dock and ryegrass did not differ (P > 0.10) but was preferred (P < 0.05) over the other forage species, while the remaining forage species did not differ (P > 0.10). In the PM, SWE and CON lambs had the same pattern of forage preference. They preferred dock and lucerne a similar (P > 0.10) amount and preferred them more (P < 0.05) than the other forage species. The SWE and CON lambs preferred (P < 0.05) ryegrass compared with plantain and chicory, and the later did not differ (P > 0.10). The SWP lambs in the PM exhibited the following order of preference: lucerne > (P < 0.05) dock = (P > 0.10) chicory > (P < 0.05) ryegrass = (P > 0.10) plantain.

The SWP lambs spent 2 to 4 times greater (P < 0.05) proportion of grazing time in chicory than CON and SWE lambs in the AM and PM, while CON and SWE lambs did not differ (P > 0.10; Table 9.5). The CON lambs in the AM spent a greater (P < 0.05) proportion of their grazing time in dock than any of the other treatments at any time point. The SWE lambs in the AM spent a similar (P > 0.10) amount of grazing time in dock as they did in the PM, but a greater (P < 0.05) proportion of time in dock compared with the remaining treatments and time points. The proportion of grazing time in dock for SWE lambs in the PM was not different (P > 0.10) compared with CON lambs in the PM and SWP lambs in the AM, but more (P < 0.05) time than SWP lambs in the PM. All lambs spent more (P < 0.05) time grazing lucerne in the PM than the AM. The SWP lambs spent a greater (P < 0.05) proportion of grazing time in lucerne compared with CON and SWE lambs, while CON and SWE lambs did not differ (P > 0.10) in the PM. In the AM, SWP lambs spent more (P < 0.05) grazing time in lucerne than the CON, while SWE lambs was

intermediate and did not differ ($P > 0.10$) from SWP or CON lambs. Within time of day (i.e. AM and PM), all lambs did not differ ($P > 0.10$) in the proportion of grazing time spent in plantain. However, when comparing the CON and SWE lambs across time, lambs spent a greater proportion of ($P < 0.05$) time grazing in plantain in the AM than all of the lambs in the PM, while SWP lambs in the AM did not differ ($P > 0.10$) from the treatments at any time. The SWP lambs had greater ($P < 0.05$) proportion of grazing time in ryegrass than CON and SWE lambs in the AM and more than the SWP lambs did in the PM. The SWP lambs in the AM did not differ ($P > 0.10$) than CON and SWE lambs in the PM. The proportion of grazing time in ryegrass by CON and SWE lambs in the AM did not differ ($P > 0.10$) from any of the treatments in the PM.

9.5 Discussion

We hypothesized that providing a plant extract to dams, would reduce the dietary neophobia of their lambs when offered the plants which made the extract. We accept this hypothesis. The SWE lambs were generally similar to the CON lambs with their grazing preference, indicating that the differences seen by the SWP lambs were due to maternal transmission of plant characteristics obtained from the SWP extract, which the lambs learned and recognized when offered those forages, and not merely due to exposure to an extract.

Providing the SWP extract to their mothers reduced dietary neophobia. This is largely seen in differences for partial preference of chicory. The SWP lambs had 279.1% and 134.5% more grazing time in chicory than CON lambs and 114.5% and 158.2% more grazing time in chicory than SWE lambs in the AM and PM, respectively. Chicory is known to be a source of plant secondary compounds such as condensed tannins and sesquiterpene lactones (Ramírez-restrepo and Barry, 2005). Both condensed tannins (Lesschaeve and Noble, 2005) and sesquiterpene lactones (Peters and Van Amerongen, 1998) are known to impart a bitter flavor. Flavors have been reported to have a hedonic value (pleasure inducing) outside of their postingestive feedbacks in ruminants (Favreau-Peigné et al.,

2013), with bitter flavors having a lower hedonic value compared with other flavors (Ginane et al., 2011). A greater hedonic value of a flavor increases the acceptance of a novel feed by naïve animals (Favreau-Peigné et al., 2013). In the current study, this may be seen in the relatively quick acceptance of lucerne by SWE and CON lambs, where they exhibited low partial preference for lucerne in the AM, but high preference for lucerne in the PM. Similar results have been reported where naïve cattle (Boland et al., 2011) and sheep (Chapter 8) that were introduced to lucerne exhibited low levels of dietary neophobia. The CON and SWE lambs had a greater acceptability of the novel lucerne in their diet compared with chicory. Accordingly, it can be concluded that lucerne has a greater hedonic value than chicory. Therefore, we believe that the greater partial preference for chicory exhibited by SWP lambs compared with CON and SWE lambs is the most compelling evidence the maternal transmission of dietary experience obtained by the plant extract.

Further support for the reduced dietary neophobia by SWP lambs can be seen in differences of partial preference for lucerne. The SWP lambs spent 168% more grazing time in lucerne compared with CON lambs in the AM and 23.6% and 37.8% more-time than CON and SWE lambs, respectively, in the PM. While less compelling than the results determined for partial preference of chicory, the differences in preference for lucerne still support lower dietary neophobia by SWP lambs.

The SWE treatment group was included as a positive control, so that it could be determined if a reduction in dietary neophobia was due to merely providing the lambs' dams with novel flavors, rather than because of exposure to particular plant components from the plant extract. It was necessary to control for this eventuality because it has been reported that exposure to novel flavors can reduce dietary neophobia, when further exposure to novel flavors occurs. For example, when lambs were offered a novel food (n = 4) each day for three days, they consumed more of the fourth novel food than the first novel food offered (Launchbaugh et al., 1997). When lambs were provided a diverse diet, they are more receptive to a new novel food than lambs without previous exposure (Catanese et al., 2012; Villalba et al., 2012). As SWE and CON lambs exhibited similar preferences among the forage species, it

can be concluded that the reduction of dietary neophobia by SWP lambs was not due to merely being used to novel flavors. Rather, the reduction in dietary neophobia by SWP lambs was due to the maternal transmission of plant components contained in the plant extract leading to dietary experience.

As seen in the current experiment, dams play a large role in developing the dietary preferences of their lambs. Dietary learning begins *in utero* (Davis and Stamps, 2004). For example, when pregnant cows were provided a high fibre diet, their offspring had a greater intake of ammoniated wheat straw later in life compared with offspring whose dams had a lower fiber diet while gestating (Wiedmeier et al., 2012). It appears that *in utero* learning is especially important in late gestation. Hai et al. (2014) provided gestating goats with *Chromonaela odorata* and found that when exposure occurred during late gestation (d 100 to 145) their lambs had lower dietary neophobia to *C. odorata* compared with lambs born to ewes that were not fed *C. odorata* during late gestation. After lambing and through to weaning, it is believed that mammals gain experience to foods through their dams milk (Provenza and Balph, 1988; Mennella, 1995; Beauchamp and Mennella, 2009). In ruminants, volatile compounds, which give foods their sensorial properties (i.e. smell and taste), consumed through their diet have been identified in milk. For example, the Besle et al. (2010) reported that ultraviolet-absorbing compounds in milk were related to cows polyphenol intake from forages. Furthermore, feeding lactating goats sulla (*Sulla coronarium* L.), which is a forage high in condensed tannins, increases the polyphenols found in their milk compared with goats consuming a grass hay (Di Trana et al., 2015). Learning about what is safe to eat from dams' from *in utero* and early life flavor experience in milk has a large effect on mammal's dietary preference later in life. Emerging from this experiment, providing dams a plant extract through gestation to weaning can manipulate young, naïve animals' dietary preference.

9.6 Implication

This experiment determined that providing a plant extract to dams reduces the dietary neophobia of their lambs when provided the plants used to make the extract. This can be seen by the

greater partial preference for chicory and lucerne in both the AM and PM by lambs whose dams were given SWP compared with CON and SWE. We conclude that it is not due to previous exposure to any plant extract, as SWE and CON lambs had similar partial preference, but rather previous exposure to plants used to create the extract. The current experiment supports previous research from our laboratory, which found that lambs provided previous experience to SWP had lower dietary neophobia compared with lambs that received no previous exposure to a plant extract or to lambs provided previous exposure to SWE (Chapter 8). Ultimately, it is concluded that exposure to forages occurs through the maternal transmission of plant characteristics. This exposure was retained by the lambs for—at least—35 days, as the extracts were only provided to the dams and the current experiment began 35 days after weaning. These results have several production implications. Firstly, providing dams extracts of plants may reduce the effect that neophobia has on the dry matter intake of naïve ruminants, thereby improving performance when animals are introduced to novel production systems. Secondly, this may benefit the landscape by reducing overgrazing of preferred forage species by increasing the incorporation of other forage species into the animal's diet. Finally, this may provide evidence for a novel management tool, where providing dams an extract of a plant may be used to increase targeted grazing of unwanted forages.

Table 9.1 Sward characteristics of spatially separated strips offered to lambs raised by hogget ewes offered either to no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP).

	Treatments				*P*-value
Item[1]	CON	SWE	SWP	SEm	Treatment
Ryegrass, kg DM/ha	4,052	4,651	5,665	741	0.34
Leaf, % of Sward	52.9	55.9	53.1	4.1	0.85
Repro, % of Sward	14.9	9.9	5.4	4.4	0.35
Weed, % of Sward	2.8	5.3	2.7	1.5	0.43
Dead, % of Sward	29.4	29.0	38.9	4.5	0.27
Chicory, kg DM/ha	3,433	3,164	3,415	276	0.75
Leaf, % of Sward	34.9	37.8	33.5	5.2	0.85
Stem, % of Sward	51.4	45.5	45.0	8.1	0.83
Weed, % of Sward	6.7	8.4	12.9	5.9	0.76
Dead, % of Sward	7.0	8.3	8.7	3.9	0.95
Plantain, kg DM/ha	3,673	3,765	2,838	326	0.14
Leaf, % of Sward	31.1	39.4	26.6	7.6	0.51
Repro, % of Sward	56.4	52.6	41.4	8.7	0.48
Weed, % of Sward	7.6	3.1	5.9	3.1	0.60
Dead, % of Sward	4.9	4.9	7.1	1.9	0.66
Lucerne, kg DM/ha	3,358	3,779	3,246	354	0.55
Leaf, % of Sward	71.8	73.7	63.6	7.0	0.57
Repro, % of Sward	—	—	—	—	—
Weed, % of Sward	17.7	16.0	24.4	4.6	0.42
Dead, % of Sward	10.5	10.3	12.0	4.0	0.95
Dock, kg DM/ha	3,206	4,279	3,989	499	0.34
Leaf, % of Sward	8.7	12.4	16.5	3.6	0.36
Repro, % of Sward	24.6	32.4	13.7	12.5	0.58
Weed, % of Sward	57.2	42.4	56.3	10.9	0.58
Dead, % of Sward	9.5	12.8	13.6	5.5	0.85

[1] Leafs were separated from stems for chicory (Stem). Lucerne was not in bloom and leaves were not separated from stems. Reproductive (Repro) for dock, plantain, and ryegrass includes stem and seed head.

Table 9.2 Chemical composition of spatially separated strips offered to lambs raised by hogget ewes offered either no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP).

	Treatment				*P*-value
Item[1]	CON	SWE	SWP	SEm	Treatment
Ryegrass DM, % as-is	37.7	35.5	37.5	1.6	0.60
CP, % DM	7.4	8.9	10.5	1.0	0.16
NDF, % DM	55.9	54.7	50.5	4.2	0.65
ADF, % DM	31.5	31.2	30.8	1.0	0.89
WSC, % DM	24.2	23.6	19.8	1.6	0.16
DMD, % DM	65.4	66.0	64.8	0.9	0.70
Chicory DM, % as-is	16.7	18.1	18.1	1.0	0.54
CP, % DM	7.3	9.0	8.9	0.8	0.28
NDF, % DM	42.5	37.4	40.5	2.4	0.34
ADF, % DM	33.7	30.6	33.4	1.4	0.27
WSC, % DM	17.7	14.9	14.8	1.9	0.49
DMD, % DM	61.2	65.1	61.6	1.9	0.35
Plantain DM, % as-is	23.1	21.4	22.2	1.0	0.50
CP, % DM	7.1	6.5	7.1	0.4	0.55
NDF, % DM	41.4	38.5	43.6	1.8	0.21
ADF, % DM	30.1	28.6	31.3	1.1	0.26
WSC, % DM	17.7	18.5	17.1	1.1	0.68
DMD, % DM	63.8	65.6	61.0	1.4	0.11
Lucerne DM, % as-is	24.8	26.6	22.8	2.2	0.49
CP, % DM	19.6	19.9	19.7	0.8	0.97
NDF, % DM	41.1	40.9	43.0	1.7	0.65
ADF, % DM	32.0	30.6	31.9	0.9	0.48
WSC, % DM	7.7	8.3	8.0	0.8	0.88
DMD, % DM	62.6	65.4	63.4	1.2	0.29
Dock DM, % as-is	29.8	26.3	28.2	2.8	0.68
CP, % DM	6.8	7.9	7.6	1.2	0.81
NDF, % DM	40.6	40.3	46.6	3.5	0.39
ADF, % DM	29.9	28.0	30.7	1.3	0.34
WSC, % DM	16.9	16.2	18.1	1.9	0.77
DMD, % DM	58.4	60.4	60.3	3.1	0.88

[1] DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; WSC = Water soluble carbohydrates; DMD = dry matter digestibility

Table 9.3 Grazing behavior of lambs grazing spatially separated strips. Lambs were raised by hogget ewes offered either to no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP).

	AM			PM				*P*-value[1]		
Item[2]	CON	SWE	SWP	CON	SWE	SWP	SEm[3]	TRT	Time	Inter.
Grazing	63.9	67.5	64.9	55.3	58.9	56.3	1.4	0.09	<0.01	0.57
Ruminating	18.0	19.3	16.0	14.7	16.1	12.7	1.9	0.41	<0.01	0.46
Idling	18.1	13.2	19.1	30.0	25.0	31.0	2.4	0.15	<0.01	0.19
Graze bouts	18.1[b]	24.3[ab]	18.4[b]	25.7[a]	26.2[a]	27.3[a]	1.6	0.19	<0.01	0.01
Bout Duration	14.8	10.9	12.8	12.6	12.6	12.1	1.0	0.26	0.46	0.02

[a-c] means within a row with different superscripts differ ($P < 0.05$).

[1] Analysis of variance *P*-value. TRT = main effect of treatment; Time = main effect of time of day, i.e. morning (AM) and afternoon (PM); Inter. = the interaction between TRT and Time

[2] Grazing, Ruminating, and Idling are percent of time doing these activities, while grazing bouts are counts, and bout duration is length of grazing bout in minutes.

[3] SEm = Standard error of the mean.

Table 9.4 Analysis of variance *P*-values for the fixed effects of the model comparing the proportion of time spent grazing in each forage strip (i.e. chicory, dock, lucerne, plantain, and ryegrass

Model Term	ANOVA *P*-value[1]
Treatment	1.00
Species	<0.01
Time	1.00
Treatment × Species	<0.01
Treatment × Time	1.00
Species × Time	<0.01
Treatment × Species × Time	<0.01

[1] Analysis of variance *P*-value. Model included fixed effects of treatment (i.e. CON, SWE, SWP), forage species (Species), and time of day (i.e. AM and PM) and the two and three-way interactions. The model tested the proportion of time spent grazing.

Table 9.5 Proportion of grazing time of lambs grazing spatially separated strips. Lambs were raised by hogget ewes offered either to no extract (CON), a seaweed only extract (SWE), or a seaweed plus terrestrial plant extract (SWP).

	AM[1]			PM[1]			
Species[2]	CON	SWE	SWP	CON	SWE	SWP	SEm
Chicory	4.3^{bC}	7.6^{bB}	16.3^{aB}	8.7^{bC}	7.9^{bC}	20.4^{aB}	4.09
Dock	63.3^{aA}	49.1^{bA}	32.5^{cdA}	34.3^{cA}	40.6^{bcA}	24.1^{dB}	4.09
Lucerne	6.2^{dBC}	10.5^{cdB}	16.6^{cB}	34.8^{bA}	31.2^{bA}	43.0^{aA}	4.09
Plantain	10.1^{abBC}	15.8^{aB}	6.0^{abB}	3.2^{bC}	0.8^{bC}	3.2^{bC}	4.09
Ryegrass	16.1^{bB}	17.0^{bB}	28.6^{aA}	19.0^{abB}	19.5^{abB}	9.3^{bC}	4.09

[a-d] Means within row with different superscripts differ ($P < 0.05$).

[1] Analysis of variance *P*-values: treatment =

[2] Percent of grazing time (%) spent grazing in each spatially separated strips.

9.7 References

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Chapter 10

Discussion

10.1 Abstract

Pastoral livestock production systems are facing considerable societal pressures to reduce environmental impact, enhance animal welfare, and promote product integrity, while maintaining or increasing system profitability. Design theory is the conscious tailoring of a system for a specific or set of purposes. Then, animals —as biological systems nested in grazing environments— can be designed in order to achieve multi-faceted goals. We argue that phytochemical rich diets through dietary taxonomical diversity can be used as a design tool for both current animal product integrity and to develop future multipurpose animals. Through conscious choice, animals offered a diverse array of plants tailor a diet, which better meets their individual requirements for nutrients, pharmaceuticals, and prophylactics. Phytochemical rich diets with diverse arrangements of plant secondary compounds also reduce environmental impacts of grazing animals by manipulating the use of C and N, thereby reducing methane production and excretion of N. Subsequently functional dietary diversity (FDD), as opposed to dietary monotony, offers better nourishment, health benefits and hedonic value (positive reward increasing 'liking' of feed), as well as the opportunity for individualism; and thereby eudaimonic wellbeing. Moreover, phytochemical rich diets with diverse arrangements of plant secondary compounds may translate in animal products with similar richness, enhancing consumer human health and wellbeing. Functional dietary diversity also allows us to design future animals. Dietary exposure begins in utero, continues through mothers' milk, and carries on in early-life experiences, influencing dietary preferences later in life. More specifically, in utero exposure to specific flavors cause epigenetic changes that alter morphological and physiological mechanisms that influence future 'wanting', 'liking' and learning of particular foods and foodscapes. In this context, we argue that in utero and early life exposure to designed flavors of future multifunctional

foodscapes allow us to graze future ruminants with enhanced multiple ecosystem services. Collectively, the strategic use of FDD allows us to 'create' animals and their products for immediate and future food, health, and wealth. Finally, implementing design theory provides a link between our thoughtscape (i.e. the use of FDD as design) to future landscapes, which provides a beneficial foodscape to the animals, and subsequently to us.

Keywords: functional dietary diversity, grazing ruminant production, welfare, environmental impact, product quality, animal design.

10.2 Introduction

Pastoral livestock producers are facing multi-faceted issues. Consumers are demanding 'cleaner and greener' production systems with 'happier' animals producing 'healthier products' for humans. Specifically, ruminants have been identified as a significant source of greenhouse gas (GHG) emissions and the removal of red meat from diets has been suggested as a mitigation of anthropogenic GHG option (Harwatt et al., 2017; Springmann et al., 2018). Consumers also have become increasingly more willing to buy animal products associated with husbandry practices that enhance animal wellbeing and welfare (Napolitano et al., 2010). Further, reductions in red meat and dairy products in human diets have been suggested to reduce negative postprandial health and collateral effects (Becerra-Tomás et al., 2016; Boada et al., 2016). Accordingly, a survey of 329 vegans, determined that 90, 69, and 47% of these people opted for a vegan diet due to concerns on animal welfare, personal health, and environmental impacts, respectively, with 82% mentioning more than one concern (Janssen et al., 2016). These concerns are multifaceted and ultimately relate to products integrity. As such, producers are pressured to respond to consumers' concerns while maintaining or increasing animal performance to remain economically viable.

Design theory is used in other fields, such as architecture, management, and finance, to address multifaceted issues (Baskerville and Pries-Heje, 2010). Design theory is defined as the preplanned

implementation framework of knowledge to generate methods and means and thereby product/s, which addresses pre-outlined single or multifaceted problems. As design theory and practice is rooted in known knowledge, it arises from pre-explored and established theories, i.e. kernel theories (Walls et al., 1992). In this context, we point out that functional phytochemical rich diets with diverse arrangements of plant secondary compounds (PSC) as a result of either taxonomical diversity or PSC rich chemical composition is a key tool to design animals that produce products with integrity. Obviously, taxonomic diversity increases phytochemical richness; however, just because a set of plant species can have complementary plant primary compounds (PPC) does not mean that they have complementary PSC. Also, different species of plants might have similar (e.g. similar types of tannins) or synergistic types of PSC (e.g. alkaloids and condensed tannins, see Provenza et al., 2007; Lyman et al., 2011; Catanese et al., 2014; Gregorini et al., 2017; Villalba et al., 2019). Accordingly, PSC diversity, as well as PPC, adds another dimension which must be functional, such that from here on we will refer to functional dietary diversity (FDD). Therefore, while likely overlapping, we separate benefits of taxonomic and PSC dietary diversity. Design theory has yet to be applied to livestock and their products; thus, and due to the multifaceted societal pressure of pastoral production systems, we argue that design theory can provide a framework to alleviate such a pressure while maintaining farms and agroecosystems profitability.

After first introducing the design concept and describing the Kernel Theories behind FDD as a tool for designing animals, we contend that FDD can be used for animal design. Moreover, we outline how FDD can answer each and all of the challenges mentioned above at the same time for the benefit of today and future pastoral livestock production systems.

10.3 Design Theory in the Context of Functional Dietary Diversity

We borrow the definition of design theory from the information systems field, as the predetermined implementation of what is known to produce something (a process or product) which addresses pre-outlined multi-faceted problems (Walls et al., 1992; Gregor and Jones, 2007). Design

theory can be split into two aspects, process and product design (Walls et al., 1992). Herein we discuss design theory for a product (i.e. animal and its product). Walls et al. (1992) describe design theory for products through a series of components with the first termed the meta-requirements, which are the designer's goals. The second component is the meta-design, defined as hypothetic artifacts to meet the meta-requirements. The meta of these two components relates to the fact that the theory does not address a single problem (i.e. requirement) or create a single artifact (i.e. design), but a cluster of problems and artifacts (Walls et al., 1992). The third component of design theory are the kernel theories (i.e. theories derived from experimentation) behind the meta-design and which can achieve the meta-requirements. The final component is testable product hypothesis (Walls et al., 1992), representing the meta-design produces a product, which meets the meta-requirements. Although design theory is used in many fields, it has not been applied to grazing ruminants. In such a context, design theory can be reworded as the implementation of established theories to grazing ruminants in order to obtain animal products which achieve pre-outlined Meta-Requirements.

Figure 10.1 depicts how the components of design theory help framework dietary diversity as a tool to design ruminant livestock. Our meta-requirements are goals, which current and future pastoral enterprises are pressured to achieve: 1) enhance animal welfare, 2) increase animal performance, 3) reduce environmental impact, 4) improve product integrity, and 5) rapidly adapt animals to evolving foodscape designs for ecosystem services. Providing FDD to ruminants is the meta-design, and the testable-design product hypothesis is that products from pastoral systems, implementing FDD will fulfill the meta-requirements. The following sections outlines specific kernel theories and explains how FDD (i.e. the meta-design) can be used for animal design to achieve our meta-requirements, shown in Figure 10.2.

10.3.1 Enhancing Animal Wellbeing and Welfare

Animal wellbeing and welfare are often wrongly used interchangeably (Barber, 2009). Animal wellbeing refers to the mental state, for example subjective interpretation of different clusters of experiences, i.e. emotions (Barber, 2009; Boissy and Erhard, 2014; Gregorini et al., 2017). Welfare instead refers to the animal state as a whole, including wellbeing and health, as well as iterative experiences with the feeding environments (Barber, 2009; Panzera, 2013). The following sections explore how animal wellbeing and welfare can be enhanced by design of diverse functional foodscapes.

10.3.1.1 Taxonomic Diversity

Eudaimonic wellbeing was proposed by Aristotle and lately discussed in diverse scientific contexts (Deci and Ryan, 2008; Nordenfelt, 2011; Harfeld, 2013; Beck and Gregorini, 2020). In our meta-design context, eudaimonic wellbeing may best apply to grazing ruminants based on function. Beck and Gregorini (2020) suggested that eudaimonic wellbeing is achieved through the pursuit of their telos; as telos has been defined as one's purpose. Therefore, when an animal is allowed to achieve/pursue their telos, eudaimonic wellbeing is enhanced, which can only occur through individual choices (Harfeld, 2013; Beck and Gregorini, 2020). This theory closely relates to the 'A Life Worth Living' (Mellor, 2016) or 'the Good Life' (Harfeld, 2013) concepts of animal welfare, which emphasize positive welfare as opposed to merely the absence of negative welfare (e.g. pain and fear). As such, experiments oriented around these theories require markers of positive welfare. Some suggested markers of positive welfare include: behavioral markers (i.e. facial expression, vocalizations, and how animals interact with their surroundings), cognitive processes, and physiological markers, but more work is required to further develop these markers (Yeates and Main, 2008). Beck and Gregorini (2020) reported FDD and choice may improve eudaimonic wellbeing by allowing the animals to display individuality, i.e. pursue their telos. Telos in grazing ruminants may be achieved individually or collectively, evidenced by individual animal and herd personalities as first suggested by (Gregorini et al., 2017) and later modelled by Moreno García

et al. (2020) [for further detail on this concepts, the reader is directed to Moreno Garcia et al. (2020)]. Genetically related personalities and grazing personalities are interesting and may provide evidence of telos in grazing animals, as it has been said that telos is intrinsic in the genetic coding of animals (Harfeld, 2013). Additionally, evidence for eudaimonic wellbeing in non-human animals is available. For example, giant pandas had lower urinary cortisol concentration when they were allowed to choose between enriched and non-enriched environments (Owen et al., 2005). Improved eudaimonic wellbeing through dietary choice may have been measured in several experiments. When lambs were allowed to choose between dietary constituents, compared to a total mixed ration; they had lower blood cortisol and lower neutrophil-to-lymphocyte ratio, indicating less physiological stress (Catanese et al., 2013). Villalba et al. (2012) also reported that lambs provided choice between dietary components compared to a mix of all the dietary components experienced less physiological stress. Individuality, and thereby telos, is related to specific genes (Boissy and Erhard, 2014). Thus, when allowed to express their individual choice and therefor pursue their telos, we hypothesis that eudaimonic wellbeing is enhanced.

Another means for FDD to enhance animal wellbeing is through improved hedonic wellbeing, which relates to pleasure, and has been suggested to be the balance between negative and positive affective states and emotions (Deci and Ryan, 2008; Beck and Gregorini, 2020). Affective states are internal conditions, including subjective interpretations of internal states (Gygax, 2017) influenced by a variety of systems stimuli (Gregorini et al., 2015; Gregorini et al., 2017). Physiologically, hedonic wellbeing is related to the dopamine, GABA, opioid, and cannabinoid systems, which are associated with eating disorders and addictions (Berridge and Kringelbach, 2008; Berridge, 2009) and meal phases and foraging behavior by ruminants (Ginane et al., 2015). As such, like in humans, there is a strong relationship between food and hedonic pleasure.

While providing pleasure, these systems are also behind meal behavior and phases: ‘wanting’, through dopamine, and ‘liking’, GABA, opioid, and cannabinoid, for specific foods and subsequently altering voluntary intake (Ginane et al., 2015). By mixing diets through choice individual requirements for

nutrients, medicines and prophylactics are better met, reducing incidental restriction and augmentation (Villalba et al., 2015; Gregorini et al., 2017). This is supported by feed conversion efficiency increments with the availability to choose dietary components freely (Nocek et al., 1986; Atwood et al., 2001; Yurtseven and Görgülü, 2004; Atwood et al., 2006). Thus increasing their ability to meet individual specific requirements, would result in a more positive internal state and would increase the hedonic value of their diets, thereby improving animal wellbeing and welfare. Ultimately, dietary choice may allow grazing managers and the animals to design 'positive-emotional' foodscapes.

10.3.1.2 Phytochemical Rich Diets and Plant Secondary Compound Diversity

Phytochemical rich diets through PSC diversity improves animal wellbeing and welfare differently than mere taxonomic diversity. Plant secondary compounds (especially phenolic compounds) are often natural antioxidants that reduce oxidative stress (Lee et al., 2017; Beck and Gregorini, 2020). Oxidative stress occurs when oxidant production outpaces the ability of antioxidant defense, which occurs following a stressor (Celi and Gabai, 2015). High concentration of circulating oxidants damage molecules, such as lipids and DNA, eliciting metabolic disorders, especially in high yielding animals transitioning to lactation (e.g. mastitis, metritis, hypocalcaemia, and retained placenta; Lykkesfeldt and Svendsen, 2007). Oxidative stress has been linked to several diseases common to other classes of livestock too, i.e. bovine respiratory disease in growing cattle (Chirase et al., 2004). As oxidative stress states can be predicted, phytochemical rich and functional diets through PSC arrangements can be ex-anti designed to alleviate oxidant loads.

Animal health relates to wellbeing and welfare. As such, freedom from disease has been listed as one of the 'five freedoms' commonly used as the basis for the ethical management of animals (Webster, 1994; Webster, 2016). Additionally, there is a direct link between oxidative damage and physiological stress. For example, isoprostanes are prostaglandin analogues which are products of oxidative damage to arachidonic acid (Montuschi et al., 2007). Prostaglandins are involved in chemical and physical injury

by increasing inflammation (Chand and Eyre, 1977). As isoprostanes are able to bind to similar receptors as prostaglandins, they cause inflammation and as such are pathophysiological mediators of inflammation from oxidative damage (Montuschi et al., 2007). Recently isoprostanes have been suggested as the most reliable markers of oxidative damage to lipid (Celi, 2011) and related with physiological stress. The isoprostane, 8 epi-prostaglandin F-2α is positively correlated with serum cortisol concentrations (Kasimanickam et al., 2018; Kasimanickam et al., 2019), providing a direct relationship between oxidative damage, increased discomfort and then reduced wellbeing. Uncomfortable experiences lead to negative emotions, reducing pleasure and subjective wellbeing. Thus by providing antioxidant support with FDD based on arrangements of PSC, we can improve wellbeing.

Additionally, PSC exhibit anthelmintic properties with animals adjusting their partial preference to consume them under gastrointestinal parasitic loads (i.e. self-medicate). Parasitized sheep preferred condensed tannins (e.g. *quebracho* [*Schinopsis quebracho-colorado*] tannins) with subsequent reductions in parasitic burdens compared to non-infected sheep (Lisonbee et al., 2009). Reductions in pathogenic bacteria also occurs through the antibiotic activity of some PSC. Ultee et al. (2002) proposed the antimicrobial mode of action of some PSC, e.g. carvacrol, to be similar to synthetic antibiotics such as ionophores (e.g. monensin by transporting H+ across the bacterial membrane and removing K+ back across the bacterial membrane; Bergen and Bates, 1984). Another example of antimicrobial activity can be seen in saponins ability to kill protozoa by forming complexes with sterols in the membrane surface (Wina et al., 2005). Reductions in protozoa improves microbial protein synthesis, slows down ruminal nitrogen cycling and reduces urinary nitrogen excretion, as well as decreased CH4 emissions (Wina et al., 2005). Lu and Jorgensen (1987) reported reductions in protozoal counts by 34 and 66% for 2 and 4% lucerne saponins additions, respectively, when added to the diet of mature wethers. In a separate experiment, saponins extracted from lucerne roots resulted in linear reductions in ruminal protozoa numbers (Klita et al., 1996). Phenolic compounds also exhibit antimicrobial effects. For example, the isoflavonoid, biochanin A, from red clover (*Trifolium pretense*) have been shown to inhibit *Clostridium*

sticklandii, which is a strain of bacteria known to be largely responsible for amino acid deamination in the rumen (Flythe and Kagan, 2010). Additionally, common phenolic acids have been shown to greatly increase *Escherichia coli* O157:H7 death rates, increasing product safety (Wells et al., 2005). Many families of PSC show antimicrobial properties and target a range of microorganisms, resulting in many subsequent effects including greater rumen fermentation and microbial metabolizable protein yield and health benefits. Collectively, these benefits will improve the internal state (e.g. nutrient supply and comfort) and sense of wellbeing, thereby welfare.

Finally, phytochemical rich diets through PSC alter animal response to stress. For example, following an adrenocorticotropic hormone challenge, sheep given either no polyphenols or one of four polyphenolic products had variable results, but overall the polyphenol had beneficial responses to immune function (Sgorlon et al., 2012). This indicates that designed PSC arrangements can improve livestock immune response to physiologically stressful events. Collectively, PSC reduce oxidative stress, parasitic and pathogenic bacteria load, and improve the immune response of stressed animals, improving their wellbeing and welfare.

10.3.1.3 Food for Thought

Improving animal wellbeing and welfare was the first proposed meta-requirement (Figure 10.1) in response to societal demands for animal wellbeing and welfare and because of the ethical 'use' of ruminants for food production. Additionally, many of the other meta-requirements are unachievable if animal wellbeing and welfare are not first addressed. As illustrated in Figure 10.2, taxonomic diversity may enhance eudaimonic wellbeing by allowing choice, leading to the display of individuality. Plant secondary compounds improve health through antioxidant defense, providing anthelmintic properties, killing pathogenic bacteria and improving immune response following physiologically stressful events. Therefore, it is hypothesized that FDD can improve hedonic wellbeing by animals being able to select

their own nutrition, flavors, and medicines leading to enhanced pleasure and positive emotions. By these means, FDD achieves the first meta-requirement.

10.3.2 Increasing Animal Production

10.3.2.1 Taxonomic Diversity

Taxonomic diversity enhances biochemical diversity providing a range of plant primary (e.g. protein, carbohydrates, and minerals) and secondary compounds. By having more choice between plant species containing different levels of plant primary and secondary compounds, ruminants tailor diets in order to meet specific individual dietary and medicine requirements (Villalba et al., 2015) and avoiding incidental restriction and augmentation (Gregorini et al., 2017). Incidental restriction is the inability to meet requirements for nutrients which are in lower concentrations in the diet and incidental augmentation is the over ingestion of particular nutrients in order to meet the requirements for nutrients that are in lower concentrations (Villalba et al., 2015). Incidental restriction reduces performance as the animal fails to meet nutrient requirements. For example, in ruminants grazing dormant pastures, protein content of forages are often below the requirements for microbial function (McCollum and Horn, 1990). As bacteria require carbon and nitrogen (N) sources, forage fermentation is reduced and thus nutrient supply from the rumen, which impairs animal performance. Incidental augmentation may likewise reduce animal performance as the removal and excretion of excess nutrients cost energy. For example, over-ingestion of N is common among ruminants in high-producing temperate pastoral systems. This excess protein is converted to urea (i.e. ureagenesis), and then either recycled back to the rumen or excreted in through urine or in milk of lactating animals (Lobley and Milano, 1997). Ureagenesis represents an energy loss and in sheep has been related to 4% of metabolizable energy intake (Waghorn and Barry, 1987). Therefore, over ingestion of nutrients reduces nutrient use efficiency and may subsequently hinder animal performance.

Another means that taxonomic diversity can increase performance is by enhancing feedstuff's hedonic value (i.e. postprandial pleasure). Hedonic wellbeing (i.e. pleasure) modulates eating behavior and rate of intake. The hedonic value of feed alters the 'liking' of feed components through the opioid and cannabinoid systems (Ginane et al., 2015). Dietary diversity increases hedonic feed value through several ways. The first is through the ingestion of different flavors, providing stimulation that motivates animals to increase intake. Villalba et al. (2011) offered one group of lambs a choice between lucerne and barley diets (75:25) either unflavored or flavored with sweet, umami, or bitter flavors compared to lambs who were offered the same diet but only one flavor profile. It was found that the lambs provided a range of flavors had greater and less variable day to day intake and tended to have greater performance compared with the lambs which had no choice. This would imply that providing a diverse diet with a range of flavors will increase energy intake by providing stimulation.

The other means by which dietary choice enhances pleasure is by animals 'formulating' their own individual diet (Provenza et al., 2003). Meeting one's requirements would lead to a better internal state, increasing positive emotions and pleasure (i.e. enhance hedonic wellbeing; Beck and Gregorini, 2020). Additionally, monotonous diets lead to incidental augmentation and thereby over ingestion of primary nutrients (e.g. protein); causing discomfort (i.e. reduced wellbeing; Provenza, 1995). Fermentation products which are associated with carbohydrates (e.g. the volatile fatty acid, propionate) has been shown to cause malaise (Provenza, 1995). Ralphs et al. (1995) reported that low-levels of propionate supplementation to their diet (0.381 Mcal of gross energy supplied) caused satiety, while high-levels of propionate addition (0.762 Mcal of gross energy supplied) caused food aversion. Villalba and Provenza (1997) reported that lambs fed increasing levels of sodium propionate (4, 8, or 12% of daily digestible energy) associated with different flavors had a preference for flavors that they were conditioned to relate to the lower additions of sodium propionate, but had significant aversions to the flavors associated with the higher levels of sodium propionate. Further, as ruminants increase ingestions of ruminal degradable protein, excessive amounts of ruminal ammonia are produced, absorbed into the

blood stream, and then converted to urea, which causes discomfort and malaise (Provenza, 1996). Over ingestion of specific primary nutrients can cause malaise and subsequent aversions, reducing food intake. This provides support for how incidental augmentation can cause discomfort and thereby reduce the hedonic feed value of specific feeds, which would result in reduced animal performance.

By improving animal wellbeing (both hedonic and eudaimonic) and welfare, animal performance improves. Poor welfare conditions with high stocking density have been shown to increase somatic cell count and reduce milk yield in dairy ewes (Caroprese et al., 2009). Likewise, following isolation lactating ewes with lower cortisol levels had 19% greater milk yield than lactating ewes with high cortisol levels (Caroprese et al., 2010). This relationship between physiological stress has been reported in growing livestock too. Lambs had lower plasma cortisol concentration when they were allowed to choose between 4 dietary components compared to lambs who were provided all of the dietary components in a total mixed ration (Villalba et al., 2012). These results were later confirmed in a similar experiment by Catanese et al. (2013), who determined that allowing lambs to choose between foods contrasting in protein:energy ratios reduced plasma cortisol concentration compared to lambs who were provided all of the foods in a total mixed ration.

Due to the relationship between welfare and performance, animal productivity and longevity has been suggested as an indicator of welfare (Barrell, 2019). Since welfare is related to performance there is economic incentive to design dietary management which allows animals to choose and display individuality thereby improving eudaimonic wellbeing, but also meeting individual requirements to increase positive emotions to improve hedonic wellbeing, can improve animal welfare and thus performance. This is supported by both the experiments of Villalba et al. (2012) and Catanese et al. (2013) who reported that lambs offered diverse diets had lower blood cortisol and several studies which have provided dietary choice to ruminants have determined performance benefits (Nocek et al., 1986; Atwood et al., 2001; Yurtseven and Görgülü, 2004; Atwood et al., 2006). Additionally, Villalba et al. (2012) found a tendency for greater rates of gain by the lambs offered choice compared with those

provided the monotonous diet. Although to our knowledge there has not been an experiment which has successfully linked providing dietary choice to reduced physiological stress and increased performance we believe that there is evidence to support this hypothesis and recommend further investigation.

10.3.2.2 Phytochemical rich diets and Plant Secondary Compound Diversity

Ingestion of PSC alters rumen function in terms of fermentation and site and extent of digestion. Bioflavex (Interquim SA, Barcelona, Spain), a flavonoid based product, increased pH in vitro (Seradj et al., 2014) and in vivo in ruminants (Balcells et al., 2012; Seradj et al., 2016) challenged with highly fermentable diets. The amelioration of acidosis was due to reductions in ruminal lactate, resulting from incremental increases in *Megaesphaera elsdenii* population, which is a lactate utilizing rumen bacteria (Seradj et al., 2016). Reviews by Waghorn and McNabb (2003) and Waghorn (2008) discussed the impact of tannins on ruminants' digestion. Tannins bind proteins, reducing ruminal protein digestion, decreasing ammonia production, and in turn increasing true protein flow to the small intestine. Tannins also reduce digestibility and fermentation rates, as well as shift fermentation patterns towards glucogenic volatile fatty acids, which reduces methane production (Waghorn and McNabb, 2003; Waghorn, 2008). Glucogenic fermentation products also increases nutrient use efficiency independently of dry matter intake, resulting in greater animal performance.

Legumes containing condensed tannins increase animal performance compared to grass based diets and other legumes like lucerne (*Medicago sativa*) and white clover (*Trifolium repens*; Waghorn, 2008). Wang et al. (1996) reported that as compared with lucerne, *Lotus corniculatus* (34 g condensed tannins/kg DM) fed to lambs without polyethylene glycol (PEG; binds extractable condensed tannins) had 8.8% greater average daily gain with 9.8% less DMI, while PEG reduced average daily gain compared with lambs without PEG. The reduced performance associated with PEG confirms that the condensed tannins were responsible for some of the production benefits. Lactating dairy cows provided with increasing levels (0, 0.45, 0.90, or 1.80% or diet DM) of a quebracho (*Schinopsis spp.*) and chestnut tree (*Castanea*

sativa) tannin extracts decreased intake linearly with tannin intake, with no reductions in milk production, thereby increasing feed efficiency (Aguerre et al., 2016). There are some experiments where condensed tannins have reduced intake and performance though. Barahona et al. (1997) reported 10% reduction of sheep intake by feeding condensed tannins. Additionally, lambs fed *Lotus pedunculatus* had low rates of growth (27 to 125-g/d). Beneficial effects of condensed tannins on performance are context dependent, i.e. source and inclusion level, as well as PSC diversity available in their foodscape.

Plant secondary compounds may also increase dry matter intake and subsequently energy intake, which then increases animal performance. Feeding capsicum (essential oil) increased dry matter intake of heifers by 10.7% (Fandiño et al., 2008). Rodríguez-Prado et al. (2012) also reported an increase in dry mater intake when feeding heifers capsicum, in this case linearly related to the level of capsicum intake with highest increase being 9.8% with 500-mg/d. Lactating dairy cows consuming cinnemaldehyde and eugenol extract blend increased dry matter intake, with a subsequent 3.6% increased milk production (Wall et al., 2014). Hence, some of the production benefits associated with PSC occur through increased dry matter and energy intake.

Isoflavonoids, another PSC are often converted to phytoestrogens in the rumen and often affect performance. For example, formononetin in red clover (*Trifolium pretense*) is metabolized to equol – estrogen- by microorganisms present in the rumen (Kelly et al., 1980), which decreased reproductive efficiency (Kelly et al., 1980; Wocławek-Potocka et al., 2013). While phytoestrogens can be negative in breeding stock, they increase average daily gain in growing animals by increasing growth hormone production (Moorby et al., 2004). These examples with phytoestrogens show how in some instances the same PSC provide benefits or can be detrimental depending on the context.

As mentioned previously, consuming PSC improve animal health through antioxidant defense, their anthelmintic, and antibiotic properties, which in turn will likely increase intake and animal performance. It is common for sick animals to show anorexia and therefore it has been identified as an indicator of discomfort, poor wellbeing and illness. When analyzing a record of 551 Holstein dairy cows

and 1,050 lactation records, it was determined that disorders such as mastitis, ketosis, and milk fever, resulted in large reduction in intake (6.7-14.7 kg) and milk production (4.1-25.7 kg) on the day of diagnosis (Bareille et al., 2003). All of these disorders have been linked to oxidative stress (Sordillo and Mavangira, 2014) and measurements of oxidative stress, when measured during dry-off (51-60 days before calving), have been used to successfully predict these disorders when the dairy cows transition back into lactation (Wisnieski et al., 2019). Likewise, in growing beef cattle (5,976 animal records) fed in a feedlot, incidence of bovine respiratory disease has been related to lower average daily gain (0.07-kg/d) and hot-carcass weight (8.16-kg) compared to those with no incidence of the disease (Schneider et al., 2009). As with transition dairy cow disorders, bovine respiratory disease incidence has been related to the oxidative stress of finishing cattle (Chirase et al., 2004). Thus, PSC provide antioxidant defense, which may be beneficial in several diseases and disorders in ruminant livestock, thereby reducing their anti-production effects. Moreover, anorexia is a common effect of gastrointestinal parasitic infection, reductions in nutrient use efficiency are key factors behind reduced performance (Parkins and Holmes, 1989), so that the anti-parasitic benefits of some PSC may alleviate these production losses. Based on these relationships, PSC provide health benefits that should then improve animal production.

10.3.2.3 Food for Thought

The second listed meta-requirement was improved animal performance, which we argued can be fulfilled, by design through FDD. The conceptual model developed in Figure 10.2 indicates the kernel theories and relationships behind providing FDD to increase ruminant performance. Taxonomic diversity may increase intake by increasing the hedonic feed value of foods, which is associated with the positive regulation on intake. Increased hedonic feeding value and eudaimonic animal wellbeing is often associated with improved animal performance. Next, PSC coming from a biochemically rich diet alters ruminal fermentation through manipulating rumen microbiome, and altering the site and extent of fermentation. Plant secondary compounds may also improve health and the welfare of ruminants.

Collectively, these benefits of taxonomic and PSC diversity can increase energy intake and improve nutrient use efficiency, improving animal performance.

10.3.3 Reducing Environmental Impact

The livestock industry represents a significant source of environmental pollutants, including greenhouse gas (GHG) emissions and nutrient losses to the waterways. In fact, the global livestock industry accounts for 14.5% of anthropogenic GHG emissions (Gerber et al., 2013). Enteric methane (CH4) and N2O emissions from manure are the largest sources of GHG from the livestock industry and represent 39.1 and 16.4% of total global livestock emissions, respectively (Gerber et al., 2013). Methane is such a large contributor to livestock GHG due to its ability to trap heat, with a 28-times global warming potential compared to CO2 (IPCC, 2013). Enteric CH4 emissions in countries which rely on intensive pastoral systems (particularly dairy) for their ruminant agriculture presents a greater source of GHG. In New Zealand, for instance, enteric CH4 accounts for 34.2% of total national GHG emissions (Ministry for the Environment, 2019). Additionally, urinary N excretion adds another significant source of environmental pollution, especially in intensive temperate pastoral operations.

The particular excess of N supplied by the base dietary forage in these intensive pastoral production systems has then become the 'limitation' to increase animal production, welfare and farm profit while reducing environmental impact. Such a limitation relates to the efficiency of nitrogen (N) utilization by ruminants, which rarely exceeds 40% (Castillo et al., 2001), meaning that at least 60% of the N ingested is not utilized to support animal production (e.g. milk, live weight gain), and is excreted, mainly (over 60%) as urinary nitrogen (UN; Kebreab et al., 2001; Gregorini et al., 2016). In dairy production systems approximately 82% of UN is discharged onto pastures (100% in meat production systems) (Oudshoorn et al., 2008; Clark et al., 2010). Due to the high N load (1000 kg N/ha) at the urine patch level, around 20–30% is leached to the waterways (Selbie et al., 2015) and 0-3.1% is transformed to N2O (a potent GHG), depending on climate and soil type (Cameron et al., 2013). Coupled with recent

reports on the associations of nitrates in drinking water and collateral risk of cancer (Schullehner et al., 2018) and the aforementioned contributions of CH4 to GHG emissions, confirm the need to explore feeding strategies to reduce, not only the amount of N flowing through grazing ruminants, but also the efficiency of rumen fermentation and methane production, in order to respond to the political and public pressures on pastoral farmers. This section will outline how FDD may be used to reduce the environmental impacts of pastoral production systems.

10.3.3.1 Taxonomic Diversity

Sustainable intensification is the goal of increasing food production through higher yields (so that more land is not converted for agricultural purposes) through more efficient use of resources (Tedeschi et al., 2015). As discussed above, taxonomic diversity can improve animal performance by increasing feed and energy intake, improving health and wellbeing, and reducing incidental restriction and augmentation. Increasing animal performance is associated with reducing days required to reach slaughter weight, which means less emission intensity (i.e. reducing GHG per kg of product produced; Capper, 2011). Emission intensity (i.e. g of CH4/unit of product produced) is an important metric for balancing food production with environmental costs (Waghorn and Hegarty, 2011). Moreover, while increased milk production increases CO2-equivalent emissions per cow from most sources of GHG emissions, the increased production dilutes the total emissions, so that emission intensity has an inverse relationship with total milk production per cow per day (Gerber et al., 2011). McAuliffe et al. (2018) determined a correlation between emission intensity and animal weight gain in pastoral beef production systems as well (r = -0.77 to -0.86). From here emerge that FDD may increase animal performance and reduce days to slaughter, thereby reducing total GHG emissions and intensity from pastoral enterprises and providing a pathway for sustainable intensification of grazing ruminant systems.

Taxonomic dietary diversity can reduce incidental augmentation and the environmental impacts by improving feed conversion efficiency. Of the total GHG emissions from livestock production, 13% is

from animal feed production (Gerber et al., 2013). Consequently, improving feed conversion efficiency will reduce feed requirements along with the amount of GHG emissions. Increased feed, i.e. nutrient use efficiency, also implies less nutrient excretion as N in the urine. Additionally, animals that were selected for better feed conversion efficiency (a.k.a. lower residual feed intake) had lower daily methane emissions (g of CH4/d; Waghorn and Hegarty, 2011). These works would imply that providing taxonomic diversity to ruminants, which increases performance levels and nutrient use efficiency, can subsequently reduce the environmental impacts of ruminant production, i.e. sustainable intensification.

10.3.3.2 Phytochemical rich diets and Plant Secondary Compounds

Plant primary, such as dietary fats (Beck et al., 2018; Beck et al., 2019) and carbohydrates (Gregorini et al., 2016; Thompson et al., 2019), but also secondary compounds, such as condensed tannins, have been explored for their potential to reduce the environmental impacts of grazing systems (Hristov et al., 2013). Several classes of PSC, e.g. phenolic compounds, have the ability to alter rumen fermentation patterns and digestion site, reducing methane emissions and urinary N excretion. Tannins are polyphenolic compounds with strong binding affinity to N, reducing protein degradation in the rumen (Waghorn, 2008). Lambs offered various forage plants with 41 g/kg of dry matter additions of a crude tannin extract (72.5% condensed tannins) had reduced crude protein digestibility, greater fecal N excretion, and lower urinary N excretion (Carulla et al., 2005). Additionally, dairy cows fed increasing proportions of a quebracho tannin extract (0, 0.45, 0.90, 1.80 % of dietary dry matter), had linearly reduced crude protein digestibility and urinary N excretion (g/d), with a linear increase in fecal N excretion (Aguerre et al., 2016).

Tannins also reduce CH4 emissions. Sheep fed 0 or 41-g of *Acacia mearnsii* tannin extract had a 13% reduction in CH4 emissions with 10% less energy lost to CH4 production (Carulla et al., 2005). Likewise, grazing dairy cows offered two levels of *Acacia mearnsii* tannins (163 or 244-g of condensed tannins/d) had a 14 and 29% reduction in methane emissions and a 10 and 22% reduction in CH4 yield by

the low and high addition level, respectively (Grainger et al., 2009). These reductions are due to reduced dry matter digestibility, and a direct inhibition of methanogenic bacteria in the rumen. Tan et al. (2011) reported that increased additions of *Leucaena leucocephala* tannins (0, 10, 15, 20, 25, and 30-mg) caused linear reduction in methanogens and protozoa numbers. Jayanegara et al. (2012) conducted a meta-analysis of 30 experiments with a total of 171 treatments and reported that for every additional 1-g tannin/kg dry matter increase there was a 0.11-mL of CH4 reduction per g of dry matter intake (R2 = 0.47).

Rumen protozoa are one of the main culprits associated with undesirable fermentation traits. For example, they engulf bacteria, decreasing microbial protein flow out of the rumen, produce metabolic H+ that is subsequently incorporated in CH4 by methanogens, and increase ruminal N cycling by increasing deamination of amino acids thereby producing ammonia as an intermediate of metabolism (Leng and Nolan, 1984; Wina et al., 2005). A *Yucca schidigera* saponins based product (Micro-Aid; DPI Global, Porterville, CA, USA) fed to steers (at either 1.1 or 2.2 g/kg of DM) reduced protozoal numbers at both levels, and increased flow of microbial N to the duodenum, (McMurphy et al., 2014). Rumen defaunation then emerges as a tool to reduce urinary N excretion and CH4 emissions (Becker, 1929; Wina et al., 2005). Zhou et al. (2011) reported that saponins derived from tea (*Camellia sinensis* L.) reduced CH4 emissions (g CH4/d) in sheep by 10.6%; while Mao et al. (2010) reported a reduction of 27.7%. These reductions were attributed to shifts in rumen protozoa populations (Mao et al., 2010; Zhou et al., 2011). While this source of saponins reduced CH4 in sheep, it had no effect when provided to cattle (Ramírez-Restrepo et al., 2016) even when provided at similar rates (~4 g/kg DMI vs ~2.4 and 3.75 g/kg DMI for Mao et al. (2010) and Ramírez-Restrepo et al. (2016), respectively).

Essential oils of plants contain a variety of phytochemicals, with 20 to 60 components found in each essential oil (Cobellis et al., 2016). The term 'essential' is derived from the word 'essence', meaning smell or taste, as these compounds are responsible for providing flavors and odors to certain herbs (i.e. thymol from thyme and oregano; cinnamaldehyde from cinnamon; Calsamiglia et al., 2007). Essential oils have

been identified as a potential fermentation modifier for reducing CH4 emissions and N excretion without compromising digestibility or intake (Cobellis et al., 2016). For example, aucubin (monoterpenoid), found in plantain (Plantago lanceolata; Gardiner et al., 2016; Mangwe et al., 2019) have been reported to reduce urinary N concentration. Part of aucubin's effect on urinary N concentration may be explained by a potential effect on rumen protozoa, as essential oils often kill protozoa (Khiaosa-ard and Zebeli, 2013). The aforementioned benefits of essential oils in vivo has been inconsistent (Cobellis et al., 2016), which relates to the variability of essential oils composition as effected by plant species and their plastic responses to the environment (Calsamiglia et al., 2007).

10.3.3.3 Food for Thought

The third meta-requirement of our FDD design is the reduction of environmental impacts associated with pastoral production systems, including enteric methane emissions and urinary N excretion. This section described the kernel theories presented in Figure 10.2. Plant secondary compound diversity can reduce environmental impact by altering ruminal fermentation. As outlined above, PSC and taxonomic diversity, i.e. FDD, can also improve animal performance, thereby reducing environmental impact per unit of product, i.e. intensity. By acting through these paths, dietary diversity may reduce the environmental impacts of ruminant production. From these works emerge again that FDD need to be thought and designed in views of phytochemical richness and PSC diversity.

10.3.4 High Quality and Healthy End-Products

Ruminant end-products for human consumption include meat and milk. Meat and milk quality is related to factors which influence eating experience (e.g. tenderness, juiciness, flavor, etc.), product appearance (e.g. color, smell, etc.), and product stability (e.g. shelf-life). The experiences of the animal, both dietary and mental, can have large implications for the quality of the final products. Furthermore, the postprandial health impacts of meat and milk may also be related to the animal's previous dietary

and mental experiences. Often, the postprandial effects of food can be seen by the subsequent increase of inflammatory markers (e.g. Li et al., 2010; Nuora et al., 2015), but also have been explored through epidemiological case studies (e.g. Bang et al., 1971). Previous work has highlighted the potential for FDD to improve product quality and human health, i.e. enhance product integrity (Provenza et al., 2019). This section will outline how FDD can improve meat and milk quality and reduce the negative health effects associated with consumption of animal products.

10.3.4.1 Taxonomic Diversity

Previously, we provided support that FDD reduces physiological stress in ruminants, as seen by reductions in plasma cortisol (Villalba et al., 2012; Catanese et al., 2013). Both long (i.e. chronic) and short-term (i.e. acute) stress can have drastic impacts on meat and milk products quality. For example, when heifers were provided shade they had a lower neutrophil-to-lymphocyte ratio compared to heifers provided no shade, indicating that heifers provided no shade experienced chronic stress. Shaded heifers had a larger proportion graded choice compared with their counterparts (Mitlöhner et al., 2002), indicating a negative effect of chronic stress on meat quality. Pre-slaughter acute stress is linked with glycogen stores depletion in muscle and thereby raising ultimate pH (≥6.0) at slaughter. The latter reduces water holding capacity, increases firmness, increases sticky texture and dark-red color of the meat (Gardner et al., 2014). As such, reductions in physiological stress by allowing dietary choice of a taxonomically diverse diet may improve end-products quality.

Meat and milk fatty acid (FA) composition largely depends on the animal's diet. Compared with grain fed systems, meat from grass-fed cattle contains greater proportions of omega-3 (n3) FA (Daley et al., 2010). Even under pasture-based diets, botanical composition of the swards influences FA profiles. Mangwe et al. (2020) reported greater milk concentrations of n3 FA for cows grazing plantain (*Plantago lanceolata* L.) and chicory (*Cichorium intybus* L.) as compared with perennial ryegrass (*Lolium perenne* L.), which can be attributed to less ruminal biohydrogenation as a result of faster outflows of digesta from

the rumen of cows grazing plantain and chicory (Mangwe et al., 2020). Similar results were previously reported by Muir et al. (2014). This highlights the potential for designing functional increases of dietary taxonomical and biochemical diversity for cows to increase —nutraceutical and prophylactic— FA concentrations in animal end-product (Provenza et al., 2015; Provenza et al., 2019).

Saturated FA have been associated with cardiovascular disease in humans (Daley et al., 2010). Red meat contains much higher concentrations of saturated FA than meat from chicken or fish (Ruiz-Núñez et al., 2016). Consequently, dietitians suggest replacing red meat with other sources of meat (Daley et al., 2010). Several works, however, contest this suggestion, suggesting that while likely a component, saturated FA content or red meat is not the major contributor to cardiovascular disease (Provenza et al., 2019). Moreover, the omega 6 (n6) to n3 ratios and n3 FA intake, has also been used to justify 'read meat intake reduction' as a health promotor (Ruiz-Núñez et al., 2016). Interest in n3 FA intake began through epidemiological studies, which concluded that Inuit peoples had lower incidence of cardiovascular disease due to greater dietary proportions of fatty fish and seal, high in n3 FA compared with other groups of people (Bang et al., 1971). Other studies have suggested that eating oily fish leads to lower cardiovascular disease (Hu et al., 2002). Subsequently, n3 FA supplements has become a common dietary recommendation for humans, despite the little evidence supporting the ability of n3 FA supplements to improve health (Albert et al., 2016; Provenza et al., 2019). In a comprehensive meta-analysis, Aung et al. (2018) reported no health benefits of taking n3 FA supplements. While this meta-analysis has been contested by von Schacky (2018), there are often clinical trials which find no benefit to n3 supplements (Sanders et al., 2011; Root et al., 2013). While high in n3, it has been suggested that there are many other compounds contained in fish, which coupled with the FA profile might increase health (Provenza et al., 2019). As such, the bioavailability of n3 FA was greater after participants were given fish rather than pills (Visioli et al., 2003). Therefore, we contend, as do others (Provenza et al., 2015; Provenza et al., 2019), that benefits from n3 FA may be seen when consumed in conjunction with synergistic compounds, found in natural sources, which increases the bioavailability of the n3 FA. This

also highlights the potential for designing functional increases of dietary taxonomical and biochemical diversity of cows to increase —nutraceutical and prophylactic— FA concentrations in animal end-product (Provenza et al., 2015; Provenza et al., 2019).

10.3.4.2 Phytochemical rich diets and Plant Secondary Compound Diversity

Plant secondary compounds often increase milk and meat quality in terms of antioxidant status (Vasta and Luciano, 2011; Villalba et al., 2019). This increment is largely associated with consumption of phenolic compounds such as flavonoids, isoflavonoids and tannins, but benefits have been shown from other PSC such as terpenoids (Brogna et al., 2014). Ingestion of PSC often increases the antioxidant status of animal products, which in turn increases shelf-life, color stability, and reduces the formation of oxidants and thereby the formation of oxidative products, such as malondialdehyde (Vasta and Luciano, 2011). Ewes fed with distilled rosemary leaf produced lambs with greater meat quality, as seen by delayed lipid oxidation, odor, and spoilage flavor compared with ewes not provided the plant extract (Nieto et al., 2010a; Nieto et al., 2011). This was also reported with ewes fed thyme (Thymus zygis ssp.) leaves, whose lambs had improved product quality (by sensory scores) and shelf life (Nieto et al., 2010b). Additionally, lambs fed quebracho (Schinopsis lorentzii) tannins had a 32% increase in total phenols and a 17% increase in antioxidant capacity compared with control lambs, improving meat color stability (Luciano et al., 2011). These experiments indicate a direct action of PSC to improve animal antioxidant status and product quality, and subsequently inhibit oxidation product formation.

Direct effects of PSC at the tissue level relate to its absorption and incorporation in tissues (Vasta and Luciano, 2011; Beck and Gregorini, 2020). We further hypothesize that oxidative defense associated with the ingestion of PSC suggests better postprandial health effects in consumers. Decreasing the formation of oxidant products such as malondialdehyde provide health benefits to humans after ingestion of meat or milk products. Oxidative by-products have been related to health issues associated with the ingestion of red meat, such as cardiovascular disease and cancer (Bowen and Borthakur, 2004;

Chiang and Quek, 2017). Further, products of oxidative damage like malondialdehyde (byproduct of lipid peroxidation) has been linked with postprandial inflammatory markers. Consumption of malondialdehyde in meat has been associated with increases in postprandial tumor necrosis factor-2 and monocyte chemotactic protein-1 concentration in the blood (Nuora et al., 2015). Therefore, by providing designed FDD, which improves the antioxidant status of the animals and their products, we can produce high quality and healthier products.

10.3.4.3 Food for Thought

The fourth meta-requirement of our design is the improvement of end-product quality to reduce the negative perceptions of adverse health effects associated with the consumption of red meat and dairy products (Figure 10.1). Physiological stress (both chronic and acute) can reduce meat quality. As taxonomic diversity can improve animal wellbeing, there may be subsequent improvements to meat quality. Moreover, providing choice of grasses and alternative forages may increase concentration and diversity of n3 FA associated with health benefits. Plant secondary compounds add to quality of animal product by reducing negative postingestive health effects. Increased antioxidant status of meat, prolongs shelf-life and inhibits the formation of oxidative by-products associated with storage and cooking, as well as postprandial inflammation, which hinders human health. As dietary diversity may increase the concentrations of beneficial FA diversity and reduce the production of products associated with negative postprandial health effects, there is a strong possibility for designed FDD to produce a product with integrity.

10.3.5 Designing animals to future food and landscapes

Finally, yet importantly, one consideration to discuss is animals' experience of designed (non-natural) food and landscapes, and how preferences and animal emotions can be 'manipulated' in early-life to the particular meta-requirement/s. As such this is our fifth kernel theory (Figure 10.2). As an example, if an animal is introduced to a new area with unfamiliar feed, they may experience neophobia

(i.e. fear of something new; Launchbaugh et al., 1997). This means that animals may not consume plants containing beneficial PSC, put in place purposely, just because they are not familiar with it. In turn, over grazing plants they are familiar with, thereby degrading the landscape, and underutilizing the manmade foodscape may occur. However, livestock can learn to overcome their neophobia, which can be done through early-life exposure, taught by their dam or peers, or influenced by management (Figure 10.3). This section outlines how grazing ruminants can be taught and/or learn to ingest beneficial plants and formulate a more functional diet, and obtain benefits described in previous sections. Moreover, the question arises, can we design an animal's future emotions and personalities for our particular benefits?

10.3.5.1 Learning from Exposure

Ruminants experience considerable amounts of neophobia when introduced to novel feedstuffs or flavors, i.e. foodscapes (Launchbaugh et al., 1997). Fear of unfamiliar feeds result from evolutionary processes by which foraging ruminants learn to consume beneficial and to avoid nutritionally poor and harmful foods (Provenza and Balph, 1987). Learning to associate post-ingestive responses to a particular feed and flavor has been discussed extensively in the literature (Provenza, 1996; Ginane et al., 2015; Gregorini et al., 2017). This learning shapes current and future dietary preferences and aversions (Provenza and Balph, 1987). Several studies with lambs have highlighted how previous experiences with specific flavors can influence acceptability of novel feeds. Sheep that had previous experience to low quality forages have a greater preference for low quality roughage compared with lambs with no previous experience and previous experiences likewise improved digestibility of the low quality forage (Distel et al., 1994). Launchbaugh et al. (1997) reported that neophobia is reduced when animals are provided a novel feed which was flavored with a familiar flavor. Additionally, repeated exposure to novel feeds increases lamb's acceptance of additional novel feeds (Launchbaugh et al., 1997; Catanese et al., 2012; Villalba et al., 2012). Finally, lambs can learn to associate post-ingestive feedbacks to their diet. For example, gastro-intestinal parasitized lambs have been shown to increase intake of condensed tannins

when they have been conditioned to associate them with their anti-parasitic effects (i.e. self-medicate; Villalba and Provenza, 2007; Lisonbee et al., 2009; Villalba et al., 2010; Villalba et al., 2014). Further, lambs have been shown to increase intake of specific flavors, if they have been paired with positive post-ingestive feedback (Ralphs et al., 1995; Villalba and Provenza, 1996; Villalba et al., 1999; Favreau et al., 2010) and reduce intake for flavors associated with nausea or toxicities (negative post-ingestive feedback; Favreau et al., 2010). Animals learn to associate positive and negative post-ingestive feedbacks to flavors and will increase or decrease intake, respectively, based on their previous experiences and what they expect to experience (unified foraging theory [e.g. predation, life history, etc.], Mangel and Clark, 1986 and preingestive cues of post-ingestive feedbacks, Favreau et al., 2010)Therefore, by incorporating the time dimension and ontology in the design of FDD animals can be taught or learn to better utilize and experience designed foodscape.

10.3.5.2 Learning from Peers

Ruminants are social animals, who follow social cues when posed with new food and landscapes (Moreno García et al., 2020). For example, when Holstein calves were provided novel feed in a social setting, they consumed more feed than animals fed individually (Costa et al., 2014). Lambs who had prior experience to a novel feed with a ewe, who was not its mother, consumed five times as much feed after weaning as lambs who were provided prior experience to the novel feeds alone (Thorhallsdottir et al., 1990). Additionally, naïve cattle alter their grazing distribution, so that they are similar to experienced cattle and this improved their grazing efficiency compared with naïve cattle without experienced peers (Ksiksi and Laca, 2000). Hence, if a naïve animal is introduced to a novel and designed FDD, neophobia may be reduced by providing timely social interactions with experienced peers. Thereby, if a production system implements specific FDD to design their animals, naïve animals can be introduced to their system and will learn from their experienced peers for their own benefit.

10.3.5.3 Learning from Mother

Mammals begin developing food preferences in utero (Davis and Stamps, 2004; Beauchamp and Mennella, 2009) with several studies reporting the importance of in utero experience on later in life preferences. Wiedmeier et al. (2012) determined that by providing pregnant cows a high fiber diet (10-fold greater neutral detergent fiber concentration), their calves had a greater intake of ammoniated wheat straw later in life compared with offspring who were provided a low-fiber diet while gestating. Additionally, pregnant ewes fed a high salt diet had offspring with altered preference and lower kidney renin activity compared with offspring born from ewes fed a low salt diet or a pasture diet (Chadwick et al., 2009b; Chadwick et al., 2009a). This in utero learning of flavors appears to be influenced by timing - stage of gestation- of flavor exposure. When gestating goats were fed *Chromonaela odorata* in late gestation, their kids had greater preference for *Chromonaela odorata* compared with when the exposure occurred during mid-gestation only (Hai et al., 2014). Therefore, by providing gestating dams a designed FDD, their offspring would have greater partial preference and lower neophobia later in their life to that FDD. In utero exposure is believed to be an evolutionary process to assist with finding adequate habitats and therefore promote survival (Davis and Stamps, 2004). Thus, by implementing animal design through FDD, offspring can be programmed to recognize, 'formulate' and enjoy the designed FDD with diverse plant species that suit the overarching meta-requirements.

Later in life, novel flavor experiences may be introduced to young livestock through their mothers' milk (Provenza and Balph, 1988; Mennella, 1995; Beauchamp and Mennella, 2009). Consumed volatile compounds are imparted into milk and play a large role in dairy products flavor. For example, polyphenols are greater in milk from goats feed diets of sulla (*Sulla coronarium* L.) compared with goats fed mixed grass hay (Di Trana et al., 2015). Additionally, Besle et al. (2010) measured aromatic compounds related to ultraviolet-absorption of milk, and determined that diet altered the type and amount of these compounds. When these compounds are ingested by the dam's offspring they may provide experience of that feed to the offspring. Several studies have shown how milk flavors alter

dietary preference later in life. For example, calves fed flavored milk replacer had greater intake of their starter ration, when it was provided with the same flavor (Morrill and Dayton, 1978). Similarly, lambs fed onion and garlic flavored milk more onion or garlic flavored feed when they received those flavors through milk (Nolte and Provenza, 1991). Hence, flavors in milk ingested by young livestock can influence their dietary preference later in life. Definitely, flavor dimensions –—as supported by their correspondent PSC— need to be added in the animal design tool box.

Finally, offspring learn consumption habits and preference through observing their dam. For example, lambs learn to self-medicate with polyethylene glycol when provided a high tannin diet if they observed their mother self-medicating early in life (Sanga et al., 2011). Learning to consume foods from their mother seems to have a larger impact than learning from animals, which are not their mother. For instance, when lambs were offered novel foods with 'mom', they consumed twice as much of that food compared with lambs exposed to the novel food with an unfamiliar non-lactating ewe (Thorhallsdottir et al., 1990). The dam-offspring relationship plays an integral role in young ruminant learning; and therefore, in their ability to survive and thrive in new foodscapes. Influence of dams on young ruminants begins in utero, continues by providing flavor familiarity through their milk, and into early life when they teach the young to develop foraging behaviors for the future. Therefore, ruminants born into designed foodscapes based on FDD, can be expected to incorporate and enjoy these plant species arrangements in their diets, thereby obtaining the designed benefits.

10.3.5.4 Food for thought

Dietary exposure begins in utero, continues through flavor experience in their mother's milk, and continues through early life experience and learning from their peers (Figure 10.3). Learning to formulate and enjoy diverse diets is key for animals to experience the nutraceutical, pharmaceutical, and prophylactic benefits of taxonomic and biochemical diversity. As such, it was included as the fifth and final kernel theory. Not only will altering preference for a designed diverse array of plants provide

benefits to the animal, it may also prevent over grazing specific plant species and areas, while reducing incidental augmentation and/or restriction with their subsequent negative impacts on performance and the environment.

10.4 Conclusions

Ruminants can be designed through the implementation of functional dietary diversity to have less environmental impacts, increase production and nutrient use efficiency, and produce a healthy and high quality product by using grazing management and nutritional knowledge and theories to promote and enhance the animal's life. Through design, we obtain the final product, which we can conduct further research on to insure that we have achieved the "Meta-Requirements" (i.e. a "Testable Design Product Hypothesis"; see Figure 10.1). This review highlights the potential for FDD to design ruminant livestock, while improving hedonic and eudaimonic wellbeing of us and our livestock. In essence, designed animals through FDD will allow us to raise happy and healthy animals, which produce healthy and environmentally clean food products, and improves animal production and therefore producer profitability. This will ultimately reconcile ruminant agriculture with the negative viewpoints of society.

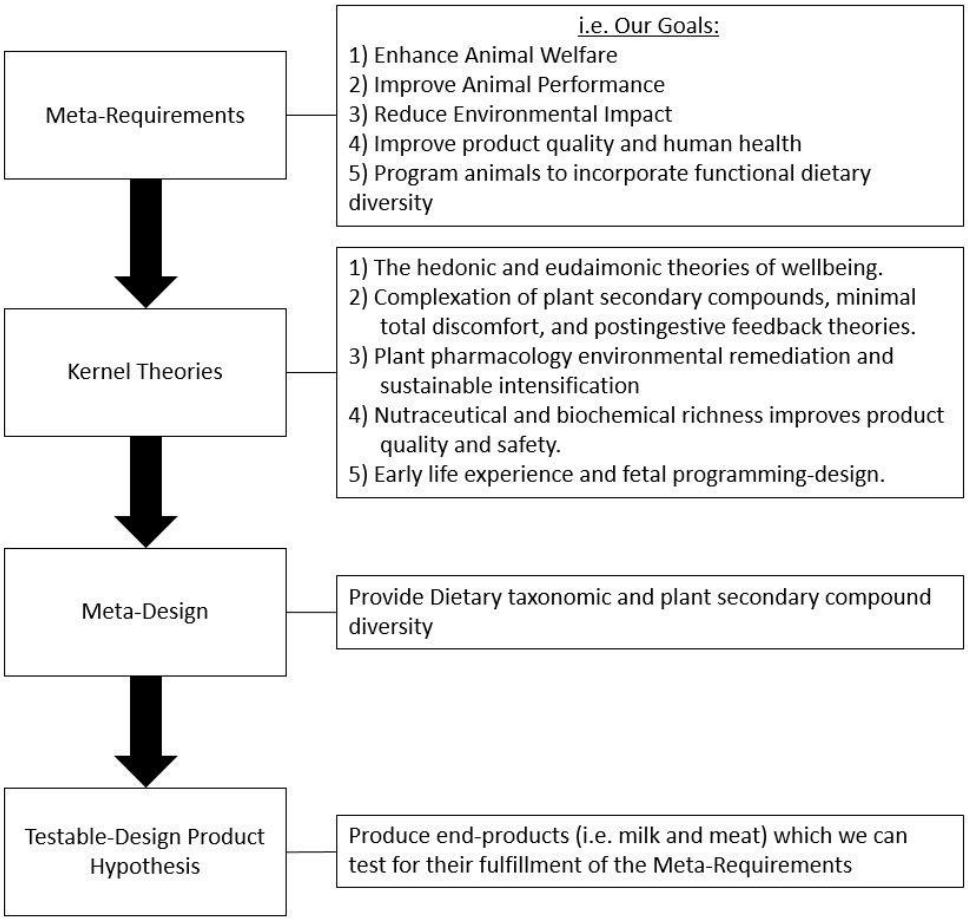

Figure 10.1 The process of design theory for products and how this process can be applied to animal design through dietary diversity (Adapted from Walls et al., 1992).

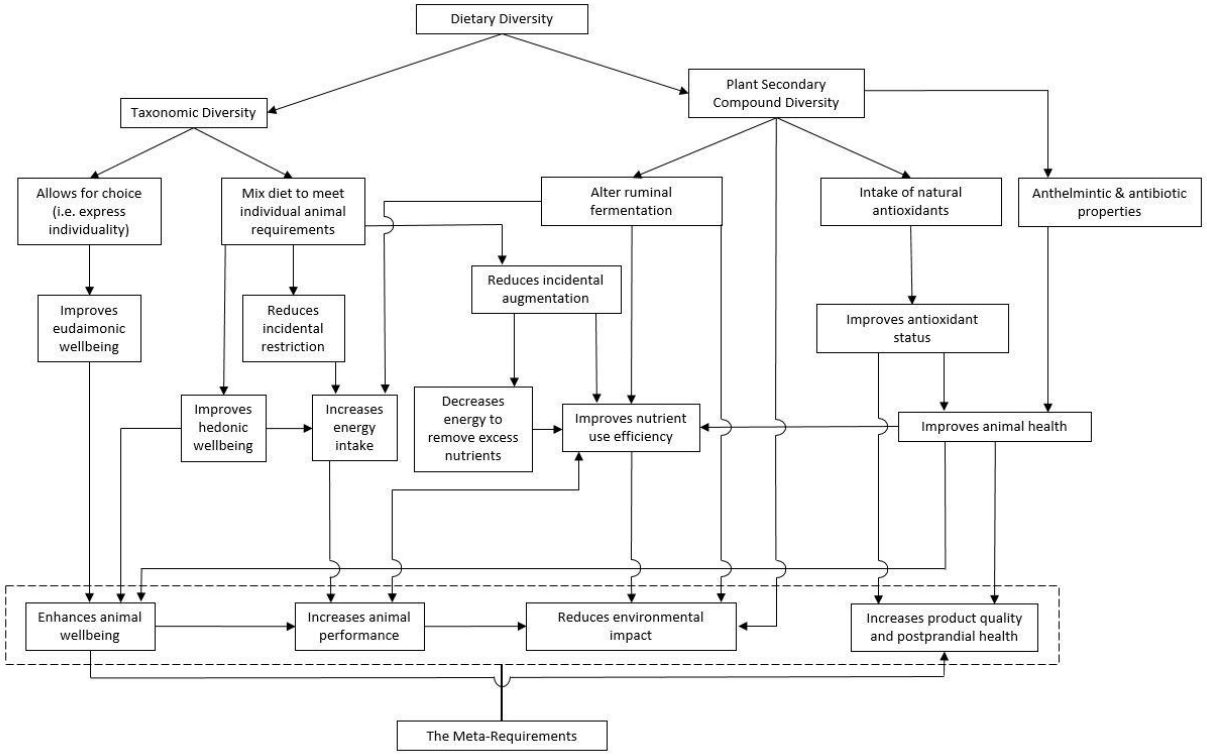

Figure 10.2 The "Kernel Theories" behind how FDD (i.e. the meta-design) can be implemented to meet our meta-requirements.

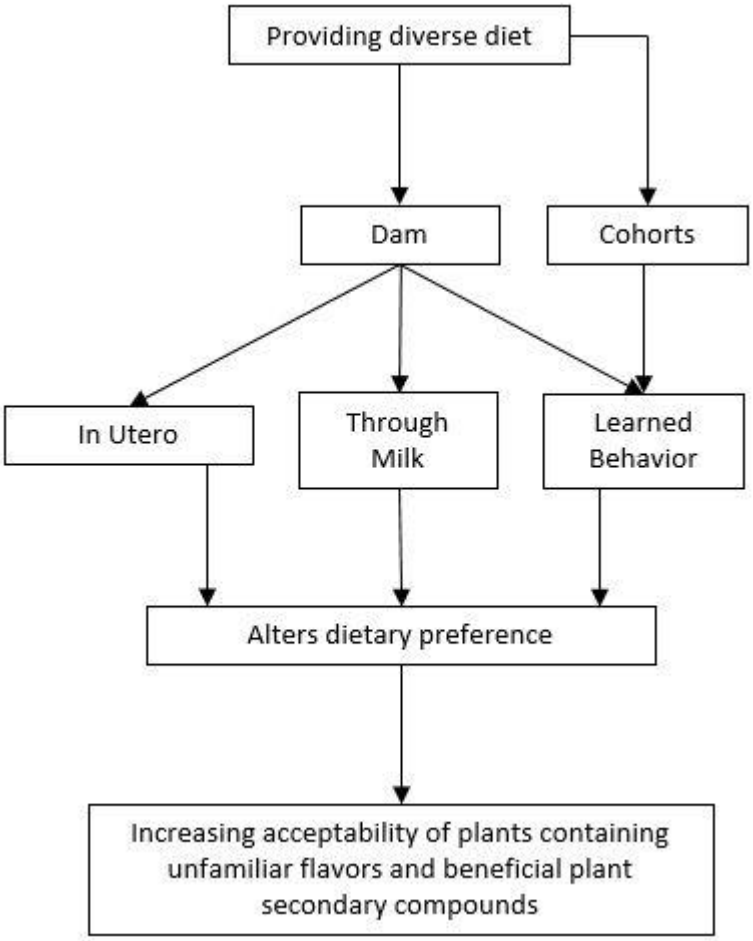

Figure 10.3 How dietary diversity can program and teach ruminants to alter their dietary preference and increase intake of plants containing beneficial plant secondary compounds.

10.5 References

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Chapter 11

General Discussion

This thesis was prepared to test the benefits of a fermented seaweed extract (SWO; Animal Health Tonic; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand) and a fermented seaweed plus terrestrial plant extract (SWP; Fortress+; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand) to ruminants. The terrestrial plants included during the extraction for SWP include equal propotions of broad-leaf dock (*Rumex obtusifolius* L.), chicory (*Cichorium intybus* L.), plantain (*Plantago lanceolata* L.), and lucerne (*Medicago sativa* L.). This thesis tested how these extracts could influence rumen fermentation, environmental impacts, oxidative stress, and their potential ability to program livestock to consume novel forage species contained in the SWP extract. It was hypothesized that the provision of rich arrangements of plant secondary compounds will: 1) alter rumen function, increasing nutrient use efficiency and reducing environmental impact in terms of methane (CH_4) production and yield, and urinary N excretion; 2) reduce oxidative stress in lactating and stressed livestock; and 3) alter foraging behaviour through providing dietary experience through these plant extracts during early life and through maternal transmission. The following sections first provide a brief introduction to why each hypothesis was tested and then discusses how the results of specific chapters answer each hypothesis.

11.1 Hypothesis One

The global livestock industry contributes 14.5% of all anthropogenic greenhouse gas (GHG) emissions, with 39.1 and 16.4% of these emissions coming from enteric CH_4 and manure nitrous oxide (N_2O) emissions (Gerber et al., 2013). The livestock industry in New Zealand represents an even larger contributor to their GHG emissions, due to the larger proportion of grazing animals. In New Zealand, 34.2% and 10.6% of their total GHG emissions are accounted for by enteric CH_4 and agricultural soils (predominantly from N_2O emission), respectively (Ministry for the Environment, 2019). When these

sources of GHG emissions are paired with the negative implication around human health effects of nitrates in the drinking water, which are a suspected carcenogen (Schullehner et al., 2018), the need to mitigate enteric CH_4 and urinary N excretion is obvious. One means that has been identified to mitigate enteric CH_4 and urinary N excretion are plant secondary compounds and plant extracts (Carulla et al., 2005; Waghorn, 2008; Tan et al., 2011; Wang et al., 2012; Beck et al., 2019). Accordingly, the first hypothesis tested during this thesis is that the plant extracts (SWO and SWP) would alter rumen function, increasing nutrient use efficiency and reducing environmental impact in terms of methane (CH_4) production and yield, and urinary nitrogen (N) excretion.

The first hypothesis was tested during chapters 3, 4, and 5. During chapter 3, it was determined that a fermented seaweed extract (SWO; Animal Health Tonic; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand) reduced *in vitro* ruminal ammonia (NH_3) production only at the lowest dose used. When this dose was extropolated to a typical dairy cow consuming 18 kg of DM per d, this dose related to 5 mL per hd per d. In chapter 4, late gestating dairy cows were orally drenched 5 mL per hd per d either water, SWO, or a fermented seaweed and terrestrial plant extract (SWP; Fortress+; AgriSea New Zealand Seaweed Ltd., Paeroa, New Zealand) it was found that total volatile fatty acid was significantly increased by 26% for the SWP treatment, and tended to increase for the SWO treatment; however, there was no effect on ruminal NH_3 or urinary N concentration. The discrepancy between what was seen *in vitro* (Chapter 3) and *in vivo* (Chapter 4), in terms of ruminal NH_3 production, was attributed to differences in dose rate, as the *in vitro* ruminal fluid was diluted with 5 parts buffer. In chapter 5, the oral drenches were increased to 100 mL per cow per d. With this dose rate, it was determined that SWP reduced daily urine N excretion by 18% and SWO tended to reduce daily urinary N excretion by 14%. When this was extrapolated out, the 18% reduction in daily urinary N excretion, may result in a 21% reduction in nitrates leached from the system. Based on these results, we accept hypothesis 1, in that SWO and SWP can reduce urinary N excretion in grazing ruminants. However, during chapters 4 and 5,

there were no differences detected for methane emissions. Therefore, we reject that portion of the hypothesis.

11.2 Hypothesis Two

Oxidative stress occurs due to the imbalance of oxidants, which include reactive oxygen metabolites, and antioxidants, such as antioxidant enzymes (i.e. superoxide dismutase), vitamin E and glutathione (Abuelo et al., 2015). This imbalance can occur during periods of physiological stress, such as from animal handling practices, or metabolic stress, including during the onset of lactation in high producing dairy cows (Beck and Gregorini, 2020). Interestingly, oxidative stress has been identified as being involved in the pathophysiology of diseases and disorders (Chirase et al., 2004; Sordillo et al., 2009; Sordillo and Aitken, 2009; Celi, 2010; Celi and Gabai, 2015). Due to the importance of oxidative stress in several disease and disorders, there has been much attention on antioxidant supplements in the scientific literature (Abuelo et al., 2015). Several plant extract products and fermented plant products have been identified as promising antioxidants for ruminants (Allen et al., 2001; Ran et al., 2019; Izuddin et al., 2020). As such, our second hypothesis was that the fermented plant extracts would reduce oxidative stress in lactating and stressed livestock.

It was determined in chapter 4, that when dairy cows during their transition from non-lactating to lactating were provided SWO and SWP, they had greater plasma total antioxidant status (TAS) and lower glutathione peroxidase (GPx) activity than the non-supplemented cows. Additionally, when sheep were offered a grain based supplement at peak lactation (Chapter 6), TAS was reduced and GPx was increased, indicating increased oxidative stress which may be related to changes in metabolic process such as oxidative phosphorylation. This negative effect of starch supplementation was ameliorated when grain was provided in conjunction with the SWO and SWP extracts. Finally, ewes, which were managed to lamb as yearlings, were offered SWO and SWP for 360 d, from their weaning to when their lambs were weaned (Chapter 7). It was determined that TAS and GPx of the ewes were not influenced by SWO and SWP two weeks prior to lambing or four weeks after lambing. Based on the level of TAS in the ewes

plasma, it was determined that the yearling ewes were effectively managing the oxidants produced and were not experiencing oxidative stress, which may explain why the SWO and SWP extracts showed no benefit to the ewes. However, one day after their lambs were weaned, it was found that the lambs born to dams provided SWO and SWP extracts had greater TAS and lower GPx activity than the lambs born to CON dams. This indicated that SWO and SWP extracts increased the maternal transmission of antioxidants to their offspring, so that oxidative stress was reduced in the newly weaned lambs. Based on these results we accept the second hypothesis.

11.3 Hypothesis Three

Naïve ruminants experience considerable dietary neophobia, meaning they avoid consuming novel foods (Thorhallsdottir et al., 1990). Classically, concerns around dietary neophobia are that it leads to significant economic losses, due to reduced intake (Ortega-Reyes et al., 1992), and also can degrade landscapes as the animals overgraze familiar foods so that they can avoid consuming unfamiliar ones (Launchbaugh and Howery, 2005). In addition to the classic concerns, there has recently been interest using functional dietary diversity which provides several production benefits, e.g. animal health and performance, but also provides several ecosystem services, e.g. reduced environmental impacts and increasing biodiversity (see Chapter 10; Gregorini et al., 2017). However, many of these principles rely on using plant species which contain specific plant secondary compounds, such as aucubin in plantain (*Plantago lanceolata* L.; Gardiner et al., 2016) or condensed tannins and sesquiterpene lactones contained in chicory (*Cichorium intybus* L.; Ramírez-restrepo and Barry, 2005). While many plant secondary compounds can have beneficial effects, they often impart bitter flavors (Peters and Van Amerongen, 1998; Lesschaeve and Noble, 2005). This is important because flavors of forages have hedonic value (i.e. pleasure inducing), with bitter flavors having lower hedonic value than other flavors (Ginane et al., 2011; Favreau-Peigné et al., 2013). Foods with lower hedonic value have been shown to induce greater dietary neophobia than foods with high hedonic value. For example, naïve animals appear to experience only minor aversion to lucerne, indicating that lucerne has a high hedonic feeding value

(see Chapter 8 and 9; Boland et al., 2011). This problem creates a new question: how can we program naïve ruminants to utilize a functional designed foodscape to obtain the planned benefits? As such, the final hypothesis tested in this thesis was that a fermented plant extract would alter foraging behaviour through providing dietary experience through these plant extracts during early life and through maternal transmission.

In chapter 8, ram lambs born during chapter 6 were provided grain supplements with either no plant extract (CON), with SWO or SWP, depending on the extracts provided to their dams, for 66 days (from weaning until the trial began). As a reminder, SWP is based on the fermented extraction of *Ecklonia radiata*, broad-leaf dock, chicory, plantain, and lucerne. The rams were then allocated to replicated diverse paddocks sown in spatially seperated strips to ryegrass (*Lolium perenne* L.), broad-leaf dock, chicory, plantain, and lucerne. Visual observations occurred the first day the rams were placed in a new break, on weeks 1, 4, and 7 of the experiment. During week 1, SWP rams spent more time grazing, had a greater number and shorter duration of grazing bouts, and spent more of their grazing time grazing chicory, than CON and SWO. The CON and SWO were similar in their grazing behavior during week 1. This indicates that early life experience to the SWP extract reduced dietary neophobia compared with the SWO and CON lambs. In chapter 9, we used ewe and ram lambs that were born during chapter 8. These lambs were precluded from consuming the CON, SWO, and SWP supplements provided to their dams and were not provided the supplements after weaning. This was done so that any dietary experience from the supplements would occur from maternal transmission. During this chapter, the lambs born to ewes provided SWP spent more grazing time in chicory and lucerne than CON and SWO lambs. These differences occurred 35 d after weaning, indicating that the dietary experience transmitted from their dams was retained for a considerable amount of time following exposure. Based on the results of chapters 8 and 9, we accept the third hypothesis of this thesis. These results indicate that plant extracts may be used to program lambs to consume novel forages.

11.4 Summary

In summary, the hypotheses tested during this thesis were chosen as they address major areas of concern for ruminant production systems —environmental impacts, animal health, and foraging ecology— and how they can be addressed using fermented plant extracts (SWO and SWP). Based on the results of chapters 3, 4, and 5 we conclude that SWO and SWP can reduce the environmental impacts by altering ruminal fermentation and reducing urinary N excretion. The effects of SWO and SWP on oxidative stress were explored during chapters 4, 6, and 7. The results of these chapters indicate that SWO and SWP can reduce the oxidative stress experienced by different ruminant species (cow and sheep) and across different physiological stages (transition from non-lactating to lactating, at peak lactation, and during physiological stressful events, such as weaning). Chapter 8 and 9, determined that providing early life experience, either directly or through maternal transmission, can reduce the dietary neophobia exhibited by naïve lambs. These results may have important implication regarding grazing ruminant production in terms of animal performance, ecosystem services (reduce overgrazing or targeted grazing of unwanted plants), or to increase the use of unpalatible forage species, which are included as part of a functional foodscape design. These experiments were conducted across different spatial and temporal scales, with different ruminant species, and with different batches of the SWO and SWP extracts. Despite the largely unknown mode of action for these products, they produce consistent effects in terms of reducing oxidative stress. This supports that these extracts provide repeatable results. Further research into the extracts mode of action is warranted. Ultimately, the results of this thesis have important implications for the ruminant livestock industry.

11.5 References

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